ABSTRACT

RNA SILENCING SUPPRESSION ACTIVITY OF THE PROTEINS ENCODED BY THE BROME MOSAIC VIRUS RNA GENOME

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The purpose of this study was to investigate the RNA silencing suppression activity of the proteins expressed by the Brome mosaic virus (BMV). For this purpose a binary, pCambia-based vector containing a GFP (Green Fluorescence Protein) gene was introduced into leaves of *Nicotiana benthamiana* (*N. benthamiana*) by agroinfiltration. The GFP gene gets transiently expressed, visualized by fluorescence activity under UV-light, and partially silenced by the RNA silencing activity (RNAi) of *N. benthamiana*.

The RNAi-based silencing in turn can be suppressed by a co-infiltrated and also transiently expressed vector, which carries a RNA silencing suppressor-gene. The suppression of RNA silencing can be visualized by a higher intensity of fluorescence under UV-light.

Two pROK2-based binary vectors, expressing the open reading frames (ORF) of either only the movement protein (MP) 3a or the coat protein (CP) were

constructed. The expression of the genes and therefore the presence of the correspondent proteins in the plant leaves was verified by WESTERN blot analysis. The T-DNA constructs expressing the two other BMV proteins 1a (BMV RNA 1) and 2a (BMV RNA 2) were received from Prof. Kao. The vector encoding the GFP-gene, as well as a construct harboring the 2b-gene, used as positive control, was received from Dr. Canto.

Every plasmid was introduced separately into *Agrobacterium tumefaciens* via electroporation. Subsequently, the agrobacteria suspensions were agroinfiltrated into *N. benthamiana*. The agrobacteria carrying the gene to be tested were always coinfiltrated with agrobacteria carrying the GFP-gene as a reporter gene. After three days, the RNA silencing suppression activity of the transiently expressed gene was determined by the comparison of the intensity of fluorescence under UV-light against a positive respective negative control.

All proteins encoded by the BMV RNA genome displayed the same intensity of fluorescence as the negative control. The positive control always showed a much higher intensity of fluorescence than any of the proteins to be tested for RNA silencing suppression activity. The results indicate that none of the BMV proteins has local RNA silencing suppression activity.

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RNA SILENCING SUPPRESSION ACTIVITY OF THE PROTIENS ENCODED BY THE BROME MOSAIC VIRUS RNA GENOME

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Chapter 1

Introduction

Brome mosaic virus (BMV)

The Brome mosaic virus is a positive sense; single stranded RNA virus (+ssRNA). It is part of the Bromovirus genus and member of the Bromoviridae family as well as the alphavirus-like supergroup.

The virions appear as icosahedral shaped particles with a diameter of about 26 nm. BMV is nonenveloped and the particles are made of 180 coat proteins. The viral genome consists of 3 RNAs: RNA 1, RNA2 and RNA3. RNA1 encodes the protein 1a and RNA2 encodes the protein 2a. These two proteins interact to form the RNA-dependent RNA polymerase (RdRp). RNA3 includes two subgenomic RNAs: sgRNA3a at the 5' end and sgRNA4 at the 3' end. SgRNA3a encodes the 3a protein, which is the movement protein (MP) and needed for cell-to-cell movement. SgRNA4 encodes the coat protein (CP).

The natural hosts of BMV are Monocotyledons, but also dicotyledonous plants like *Nicotiana benthamiana* and some Chenopodium species can be infected [1].

RNA interference

The term RNA interference (RNAi) or RNA silencing refers to a mechanism of gene silencing where small RNAs with a length of 21 to 30 base pairs play a central role. These small RNAs can be classified, due to their place of origin, as short interfering RNAs (siRNAs) or microRNAs (miRNAs) and determine the specificity of the RNA silencing mechanism. The silencing of the genes is achieved by RNA cleavage, translational repression or methylation of DNA or chromatin.

The enzyme Dicer accomplishes the first step of RNA silencing. Dicer is a type three endonuclease, which cuts RNA strands to a size of 21 to 30 base pairs. MiRNAs are cut to their length from single stranded RNAs (ssRNA) with a stem loop structure. These RNAs are encoded by genes of the cell itself and their silencing activity serves regulatory functions. SiRNAs on the other hand are derived from longer RNA molecules that do not originate from the cell. They can stem from a virus, an introduced plasmid or any artificially made double stranded RNA (dsRNA). After Dicer has truncated them, they follow the exact same pathway of RNA silencing as miRNAs [2].

The small RNAs interact with the RNA induced silencing complex (RISC). When double stranded small RNA attaches to the RISC it gets denatured and one strand of the small RNA, called passenger strand, gets discarded. The other strand, called guide strand, stays and it determines the specificity of the RISC.

Together with the guide strand the RISC is able to silence RNA by three ways, which comprise methylation of chromatin, RNA cleavage or translational arrest of target mRNAs. RISC includes, besides other several proteins, a member of the Argonaute family. The Argonaute protein (AGO) contains a Piwi domain that possesses the catalytic ability to cleave mRNAs, that pair with the guide strand, and thereby silence the gene the mRNA was transcribed from. If the guide strand does not match perfectly to the target mRNA it will not be cleaved but translation will be inhibited instead.

The third way of RNA silencing led by both RISC and the small RNAs is the methylation of DNA or chromatin. In this case the Pol IV subunit b (NRPD1b) is recruited to the complex and the DNA cytosine residue or histone proteins or both are methylated. This methylation stops the transcription of the specific gene and thereby silences it [3].

Furthermore, plants and fungi can express an RNA dependent RNA polymerase (RdRp), that is a highly efficient tool of the RNA silencing pathway. It is able to amplify short dsRNAs after the RISC complex got attached to the target RNA. Such amplified dsRNAs can then fuel the Dicer enzyme [4].

RNA silencing suppression

RNA silencing is not only a self-regulatory mechanism of gene expression (by miRNAs), but also a host defense mechanism (by siRNAs) that will silence viral genes and prevent viral replication. In order to overcome this problem, viruses have developed a counter defense strategy: the viral suppression of RNA silencing. Over 40 viral suppressors of RNA silencing (VSRs) (shown in the following table) have already been described to date, and it is suspected that each plant virus at least encodes one or more VSRs [5; 4].

Table [1]: Table of RNAi suppressors

Virus group	Virus name	RNAi suppressor	Motif implicated in RNAi suppressor activity
Positive-strand RNA viruses in plants			
Aureusvirus	Pothos latent virus	P14	dsRNA binding
Carmovirus	Turnip crinkle virus	CP	
Closterovirus	Beet yellows virus	P21	dsRNA binding
Closterovirus	Citrus tristeza virus	P20	
Closterovirus	Citrus tristeza virus	P23	
Closterovirus	Citrus tristeza virus	CP	
Closterovirus	Grapevine leafroll-associated virus-2	P24	
Closterovirus	Beet yellow stunt virus	P22	
Crinivirus	Sweet potato chlorotic stunt virus	P22	
Crinivirus	Sweet potato chlorotic stunt virus	RNase3	RNaseIII
Comovirus	Cowpea mosaic virus	Small CP	
Cucumovirus	Cucumber mosaic virus	2b	dsRNA binding
Cucumovirus	Tomato aspermy virus	2b	dsRNA binding
Furovirus	Soil-borne wheat mosaic virus	19K	Cysteine-rich protein
Hordeivirus	Barley stripe mosaic virus	γb	Cysteine-rich protein
Pecluvirus	Peanut clump virus	P15	Cysteine-rich protein
Polerovirus	Beet western yellows virus	P0	
Polerovirus	Cucurbit aphid-born yellows virus	P0	
Potexvirus	Potato virus X	P25	
Potyvirus	Tobacco etch virus	Hc-Pro	
Potyvirus	Potato virus Y	Hc-Pro	
Potyvirus	Turnip mosaic virus	Hc-Pro	

Continued on the following page

Table [1] continued

Virus group	Virus name	RNAi suppressor	Motif implicated in RNAi suppressor activity
Positive-strand RNA viruses in plants			
Sobemovirus	Rice yellow mottle virus	P1	
Tobamovirus	Tobacco mosaic viruses	P130	
Tobamovirus	Tobacco mosaic viruses	P130	
Tobravirus	Tobacco rattle virus	16K	Cysteine-rich protein
Tombusvirus	Tomato bushy stunt virus	P19	dsRNA binding (Prefer 19-nt RNA duplex)
Tombusvirus	Cymbidium ringspot virus	P19	dsRNA binding (Prefer 19-nt RNA duplex)
Tymovirus	Turnip yellow mosaic virus	P69	
Vitiviruses	Grapevine virus A	P10	
Negative-strand RNA viruses in plants			
Tenuivirus	Rice hoja blanca virus	NS3	
Tospovirus	Tomato spotted wilt virus	NSs	
Double-stranded RNA viruses in plants			
Phytoreovirus	Rice dwarf virus	Pns10	
DNA viruses in plants			
Begomovirus	Tomato leaf curl virus	C2	DNA binding, NLS
Begomovirus	TYLCCNV-Y10 Y10β	βC1	DNA binding, NLS
Begomovirus	African cassava mosaic virus (KE)	AC2	DNA binding, NLS, AD
Begomovirus	EACMCV, ICMV, TGMV	AC2	DNA binding, NLS, AD
Begomovirus	Mungbean yellow mosaic virus	AC2	DNA binding, NLS, AD

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Table [1] continued

Virus group	Virus name	RNAi suppressor	Motif implicated in RNAi suppressor activity
Positive-strand RNA viruses in plants			
Begomovirus	African cassava mosaic virus (CM)	AC4	miRNA binding (Single-strand mature miRNA)
Curtovirus	Beet curly top virus	L2	Protein binding
Positive-strand RNA viruses in animals			
Nodavirus	Flock house virus, nodamura virus, Striped jack nervous necrosis virus, Greasy grouper nervous necrosis virus	B2	dsRNA binding
Negative-strand RNA viruses in animals			
Orthomyxovirus	Influenza virus A	NS1	dsRNA binding
Orthobunyavirus	La Crosse virus	NSs	
Double-stranded RNA viruses in animals			
Orthoreovrivus		σ3	dsRNA binding (Prefer dsRNA longer than 30 nt)
Retroviruses in animals			
Lentivirus	HIV-1	Ta t	
Spumavirus	PFV-1	Tas	
DNA viruses in animals			
Adenovirus	Adenovirus	VA1 RNA	Dicer binding
Poxvirus	Vaccinia virus	E3L	dsRNA binding
Mycovirus, double-stranded RNA virus			
Hypoviridae	Cryphonectria hypovirus 1	P29	

Plant RNA silencing suppressor groups

Plant RNA silencing suppressors can be divided into three groups. The first group of VSRs is able to stimulate virus accumulation in inoculated protoplasts. The second group consists of VSRs that are fundamental for virus cell-to-cell movement, but are not necessarily needed for the single cell virus accumulation. Last but not least, the third group of VSRs enables viruses for long distance movement or intensifies disease symptoms (or both), although it is not fundamental for viral replication and cell-to-cell movement.

Most VSRs are viral proteins that target one or more host proteins of the RNA silencing pathway or of the small RNAs itself and thereby interrupt the pathway. For example, some VSRs of the first group above attach to the AGO component of the RISC. It gets degraded and inoperable. Therefore AGO cannot cleave the target RNA or load small RNAs to the RISC complex anymore. VSRs of the second group especially target small RNAs [6].

Chapter 2

Materials and Methods

Construct

Two constructs were constructed, one containing the CP ORF of RNA3 of BMV and one containing the whole RNA3 sequence. Both inserts were ligated into the 35S promoter-polylinker-NosTerminator T-DNA cassette of pROK2-based binary vectors. The pROK2 vector was received from Dr. Tomás Canto (Centro de Investigaciones Biológicas, CIB, CSIC, Madrid 28040, Spain).

CP construct

In order to construct the CP encoding sequence of BMV into pROK2, the corresponding sequence was amplified by polymerase chain reaction (PCR) using the pB3 plasmid as a template [7]. Taq I polymerase purchased from Promega was used in all PCR reactions. For cloning purposes, the upstream primer homologous to nucleotides 1230-1264 of the BMV RNA3 sequence (5-GCCATCGGATCCGTTCAGC GTATTAATAATGTCGACTTCAGGAACTGG-3), with added BamHI and additional 5 nucleotides terminal sequences, were used in conjunction with a downstream primer that was complementary to nucleotides 2069-2111 of the RNA3 sequence (5-GCCAT CCCCGGGGTGGTCTCTTTTAGAGATTTACAGTGTTTTTCAACACTGTACGG-

3), preceded by a SmaI sequence and five additional nucleotides at the 5' end. The amplified PCR cDNA product then was digested with BamHI and SmaI restriction enzymes and was ligated into pROK2 that was linearized with the same enzymes. T4 DNA ligase purchased from New England BioLabs was used for ligation. The construct was multiplied in Escherichia coli strain DH5α cells, under kanamycin (50μg/ml) selection. After multiplication the construct was introduced into *Agrobacterium tumefaciens* cells by using electroporation with 2.4 kV, 200 Ω and 25 μF with the Gene Pulser II Electroporation System from BIORAD and grown in LB medium with 50 μg/ml kanamycin and 50μg/ml carbenicillin. The *A. tumefaciens* strain C58C1 was provided by Dr. Tomás Canto (Centro de Investigaciones Biológicas, CIB, CSIC, Madrid 28040, Spain).

RNA3 construct

In order to construct the entire BMV RNA3 sequence into pROK2 vector, the corresponding sequence was amplified by polymerase chain reaction (PCR) using the pB3 plasmid as a template [7]. Taq I polymerase purchased from Promega was used in these PCR reactions. For cloning purposes, an upstream primer that was homologous to nucleotides 1-35 of the RNA1 RNA3 BMV sequence (5-GCCATCGGATCCGGTAAAATACC AACTAATTCTCGTTCGATTCCGGCG-3), with added BamHI and additional five 5'-terminal nucleotides, was used in conjunction with a

downstream primer complementary to nucleotides 2069-2111 of the RNA3 sequence (5-GCCAT CCCCGGGGTGGTCTCTTTTAGAGATTTACAGTGTTTTTCAACACTGTACGG-3), preceded by a SmaI sequence and five additional nucleotides at the 5' end. The amplified PCR product was digested with BamHI and SmaI and ligated into pROK2 that was pre-linearized with the same enzymes. Again, T4 DNA ligase purchased from New England BioLabs was used for ligation. The construct was multiplied in Escherichia coli strain DH5α cells, under kanamycin (50μg/ml) selection. After multiplication the corresponding plasmid construct was introduced into *A. tumefaciens* via electroporation with 2.4 kV, 200 Ω and 25 μF and grown in LB medium containing 50μg/ml kanamycin and 50μg/ml carbenicillin.

GFP and 2b constructs

Dr. Tomás Canto provided the binary, pCambia-based vectors containing the GFP gene as well as the pROK-based vector containing the 2b gene as a generous gift. The 2b gene is a well-known RNA silencing suppressor from Cucumber mosaic virus (CMV).[8]

RNA1 and RNA2 constructs

The T-DNA-based constructs containing a full-length RNA1 or RNA2 sequences were received from Prof. C. Cheng Kao (Department of Biochemistry & Biophysics, Texas A&M University, College Station, Texas). The vector used in these constructs is named as pCB302.

Transient expression of genes in plant tissue

A. tumefaciens cultures were incubated on a shaker for approximately 11 hours in Luria-Bertani (LB) medium containing 50μg/ml kanamycin and 50μg/ml carbenicillin at 28°C and 170 rpm. After low-speed centrifugation (25°C, 10 min., 6000 rpm) the supernatant was rejected and the cells were resuspended in an agroinfiltration buffer (200μM acetosyringone, 10mM MES, pH 6.8 and 10mM $MgCl_2$). The suspension was further diluted with the agroinfiltration buffer to reach the OD (OD_{600}) of 0.2 at 600nm. The final suspension was then agroinfiltrated into *N. benthamiana* leaves by gently pressing the tip of a 1 ml syringe, without needle, at the bottom side of the leaves and injecting the suspension till the growing patch in the leaves achieves about 3cm in diameter. The leaves of *N. benthamiana* used for agroinfiltration were always fully expanded. In a separate experiment the Chenopodium quinoa leaves were injected in a similar manner.

Two abreast sites were infiltrated on each leaf, one located to the left of the central leaf vein and one located to the right. At most two leaves per plant were infiltrated. In each site a culture of different combined *A. tumefaciens* suspensions harboring different T-DNAs was injected. Each combined suspension contained a GFP reporter gene from a binary vector. With this GFP reporter gene either an empty binary vector pROK2 (as a negative control) or a vector harboring the 2b gene from CMV (as a positive control) was combined on the left side of each leaf. The right side of each leaf was infiltrated with another vector expressing a protein or protein combination to be tested for its RNA silencing suppression activity and the GFP reporter gene-vector. To monitor the effect of the protein or protein combination on the intensity of GFP fluorescence, the leaves were illuminated after 3 days post infiltration by using a Black Ray long wave (365nm) UV lamp (UVP, Upland, CA, U.S.A.). The photos were taken with an Exilim digital camera from Casio (10.1 mega pixels, EX-S10).

In further experiments barley leaves (*Hordeum vulgare)* were treated in a similar manner, to determine if barley is an appropriate host plant for the agroinfiltration assay. For this experiment one part of fully expanded, but singular leaves were infiltrated like described before and another part was angular cut and incubated standing in the agrobacterium suspension.

Protein analysis

For protein analysis, about 0.5g of infiltrated leaf tissue was ground to a powder in liquid nitrogen with a prechilled mortar and pestle. The total proteins were then extracted with the following extraction buffer (4 ml per 1g tissue): 0.1 M TrisHCl (pH 8), 10 mM Ethylenediaminetetraacetic acid (EDTA), 0.1 mM LiCl, 2% sodium dodecyl sulphate (SDS), 2% phenylmethanesulfonylfluoride (PMSF). Then the samples were heated for 10 minutes at 93°C and separated by electrophoresis in a sodium dodecyl sulfate-polyacrylamide electrophoresis 12% gel. The ColorPlus Prestained Protein Ladder, Broad Range (10-230kDa) from NEW ENGLAND BioLabs was used as a size standard. Subsequently, the Trans-Blot Turbo Transfer System from BIORAD was used to blot the proteins onto a Polyvinylidene Fluoride (PVDF) Membrane with 2.5A and up to 25V for 7minutes. The CP and 3a proteins were detected immunologically by using an anti-Brome mosaic virus polyclonal antibody from Agdia and an anti-BMV-3a, rabbit-antiserum antibody from K. Mise (Kyoto University), respectively. For detection of the bound primary antibody, an ECL anti-rabbit IgG, horseradish peroxidase-linked species-specific whole donkey antibody (Amersham) was used. The enhanced chemiluminescent (ECL) substrate for the linked horseradish peroxidase (HRP) enzyme (purchased from Thermo SIENTIFIC) was used to visualize the bands of interest.

Chapter 3

Results

Cloning and transient Agrobacterium-based protein expression

The CP construct was achieved by cloning. For this purpose the CP ORF was first amplified by PCR from the RNA3 of BMV, figure [1], using primers that cover only the CP ORF.

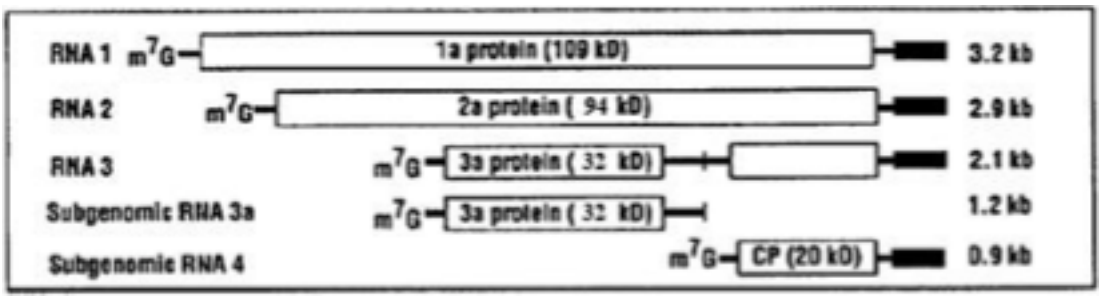

Figure [1]: Map of BMV genome. The genome consists of 3 RNAs., RNA1, RNA2 and RNA3 [9]

A BamHI terminal sequence had been added to the upstream primer used for this PCR. In the same manner a SmaI terminal sequence had been added to the downstream primer.

In the second step, the amplified PCR cDNA product and the pROK2 plasmid were digested with BamHI and SmaI restriction enzymes. The pROK2 plasmid

carries a 35S promoter-polylinker-NosTerminator T-DNA cassette and the BamHI and SmaI cutting side as outlined in figure [2].

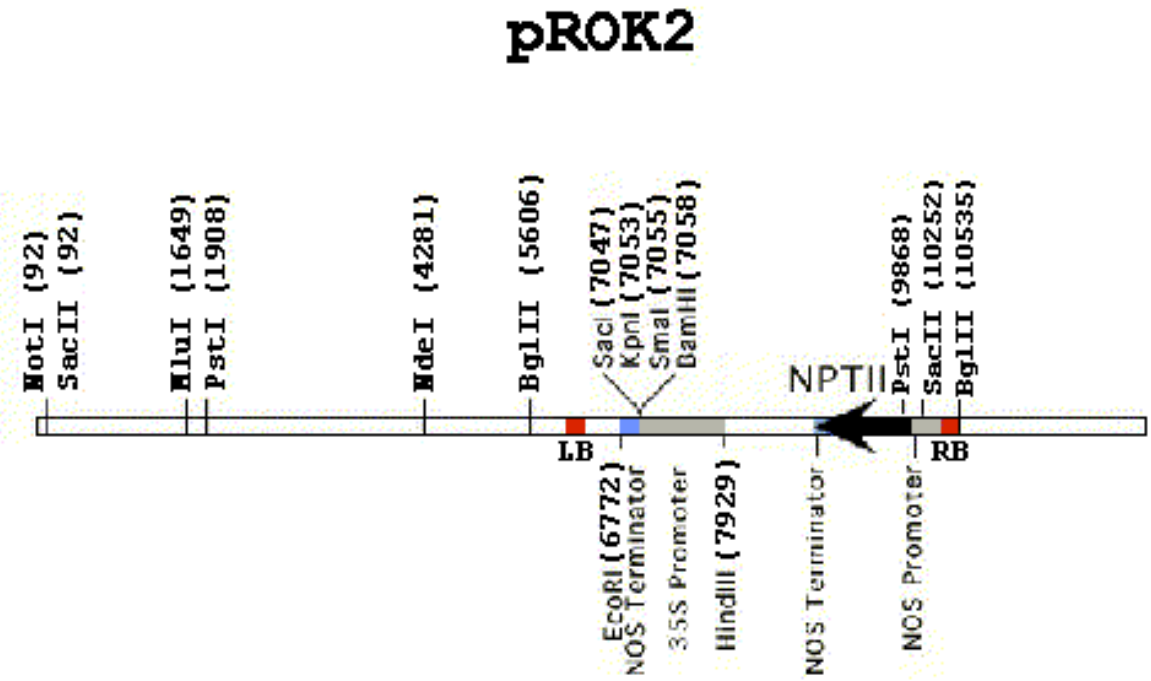

Figure [2]: Map of the binary vector pROK2 carrying a 35S promoter-polylinker-NosTerminator T-DNA cassette, Salk Institute Genomic Analysis Laboratory

The digested PCR product was then ligated into the binary vector pROK2. The final construct, as illustrated in figure [3], was subsequently electroporated into the agrobacteria strain C58C1.

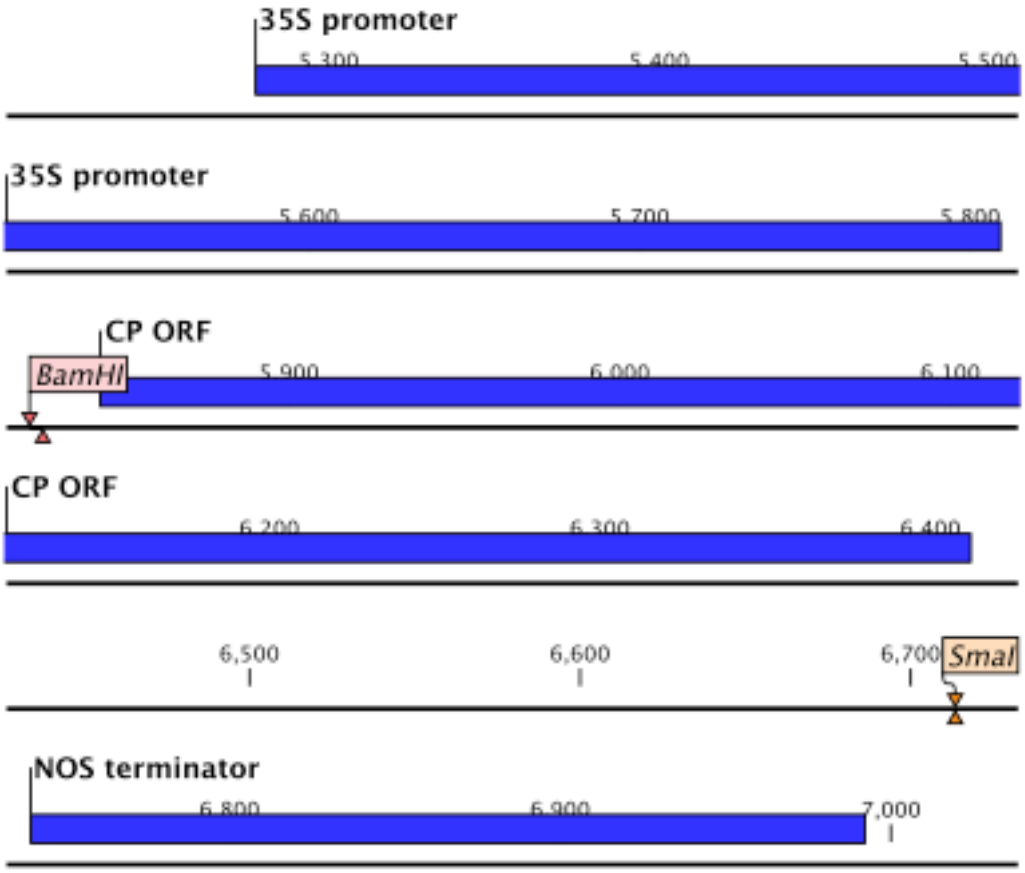

Figure [3]: CP construct, CP ORF was cloned between the SmaI, BamHI cutting sites of the pROK2 vector.

The RNA3 construct was achieved by a similar cloning procedure. RNA3 was first amplified by PCR from RNA3 of BMV, figure [1], using primers covering the total RNA3. A BamHI terminal sequence had been added to the upstream primer

used for this PCR. In the same manner a SmaI terminal sequence had been added to the downstream primer.

In the second step, the amplified PCR cDNA product and the pROK2 plasmid were digested with BamHI and SmaI restriction enzymes. The digested PCR product was then ligated into the same binary vector pROK2.

The final construct, as illustrated in figure [4], was as well subsequently electroporated into the agrobacteria strain C58C1.

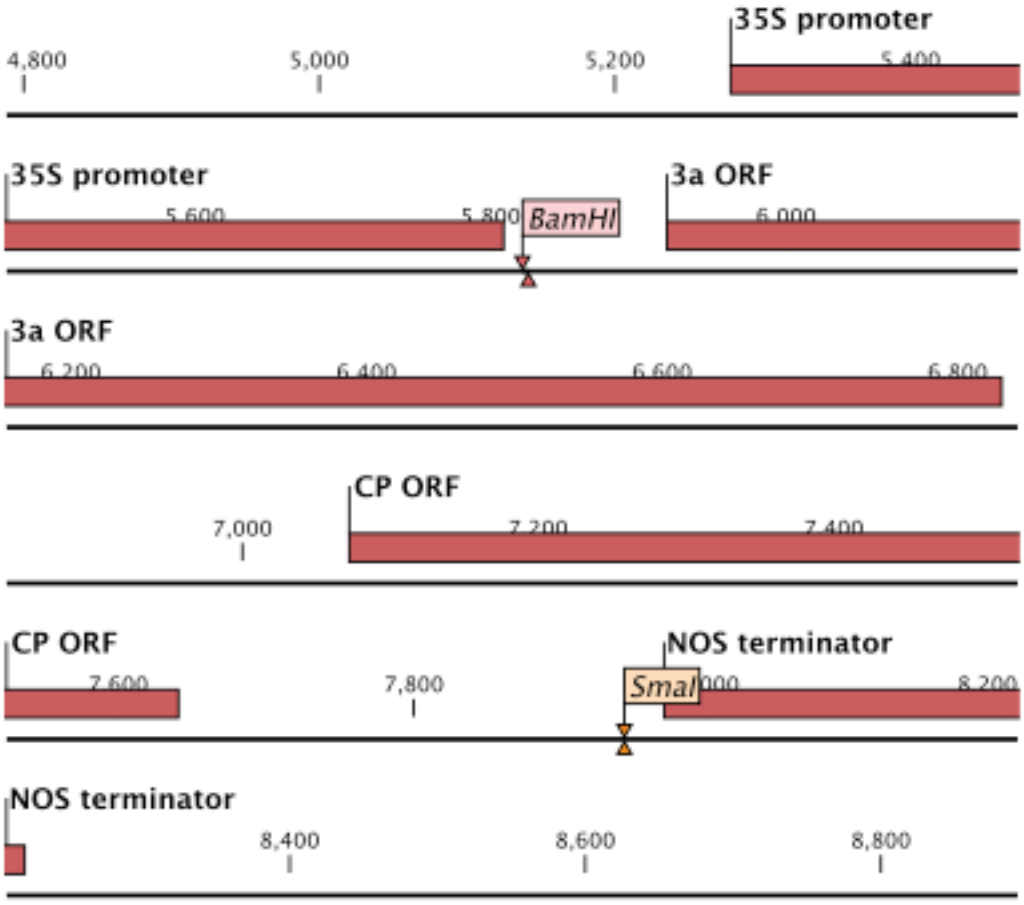

Figure [4]: 3a (movement protein) construct, RNA3 was cloned between the SmaI, BamHI cutting sites of the pROK2 vector.

A similar cloning procedure was used for the received 2b construct (from Dr. Canto), where the same pROK2 plasmid was processed, figure [2]. For PCR a different template was used. To construct the 2b gene into the pROK2 plasmid the

RNA2 of CMV was amplified using primer that cover only the 2b ORF, as represented in figure [5].

Figure [5]: The CMV RNA genome consists of 3 RNAs, RNA1, RNA2 and RNA3. [10]

For the GFP construct a different pCambia-based plasmid was used, figure [6].

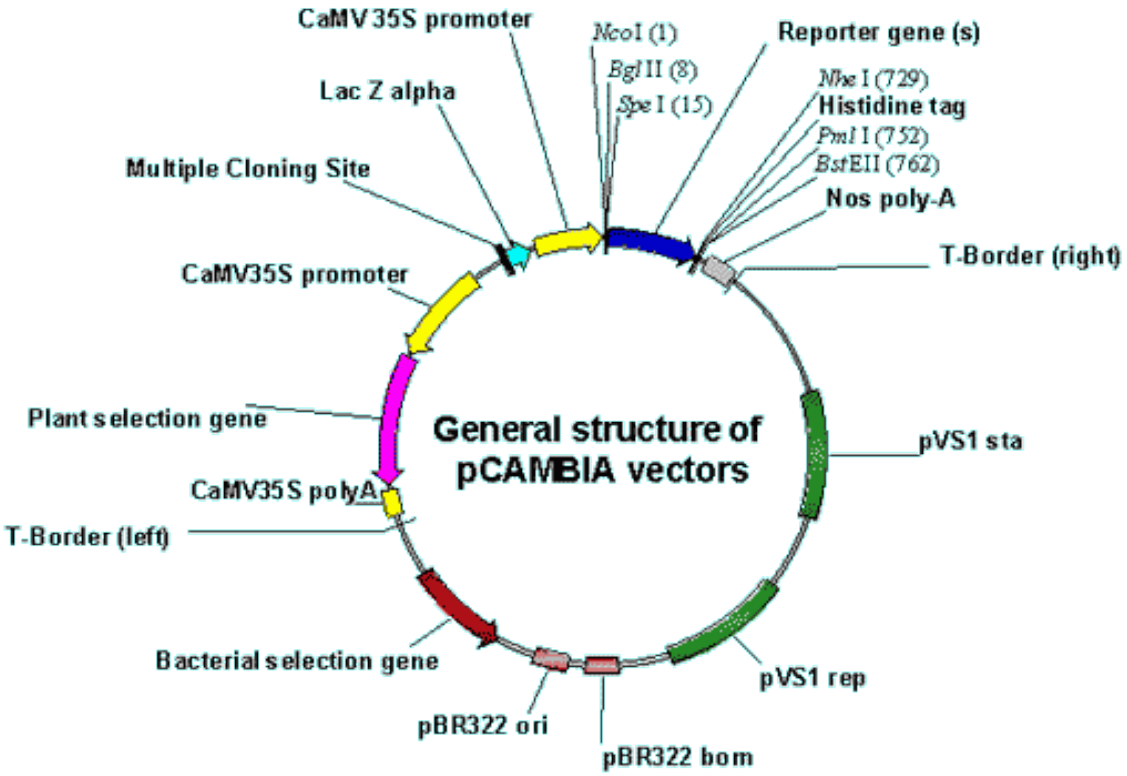

Figure [6]: binary pCambia vector, GFP-gene was used as a reporter gene, cambia

In this case the GFP gene was used as the Reporter gene in the pCambia vector.

The GFP gene was originally cloned from the genome of the cnidarian, Aequorea Victoria, which naturally expresses the green fluorescence proteins (GFP) [11]. After

cloning it into the pCamiba vector it was electroporated into agrobacteria strain C58C1.

For the RNA1 and RNA2 construct, received from Prof. C. Cheng Kao, a binary pCB301 vector was used. RNA1 and RNA2 of BMV, figure [1], were cloned in the T-DNA region between the NcoI and XbaI restriction sites [12]. The final construct, illustrated in figure [7], was subsequently electroporated into agrobacteria strain C58C1.

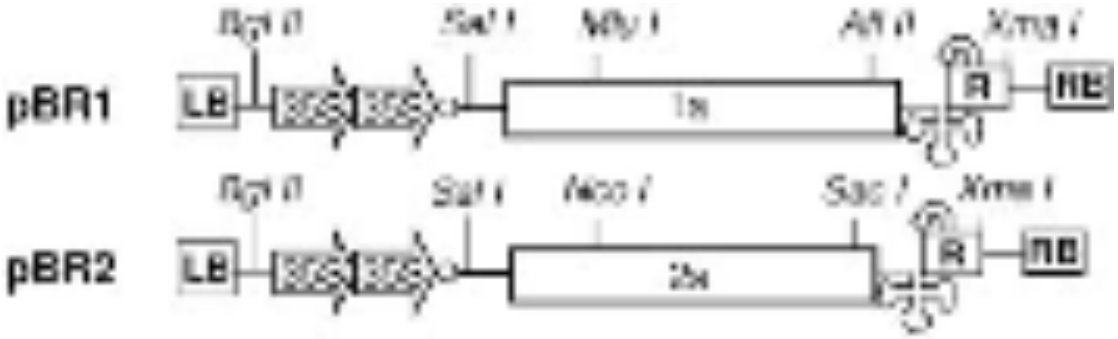

Figure [7]: RNA1 and RNA2 constructs, expressing the 1a and the 2a proteins respectively, of BMV. Constructs were received from Prof. Kao [12].

Agrobacteria are able to transfer the construct into plant cells after infiltration. *Agrobacterium tumefaciens* is a soil phytopathogenic gram-negative bacterium that interacts with plant cells. It attaches to their surface and transfers T-DNA into the cell cytoplasm. A protein complex provided by the bacteria protects

the T-DNA. These proteins also convey the complex to the nucleus where the DNA is released and gets integrated into the chromosomes of the plant.

As part of the plant genome the DNA will be transcribed and subsequently translated. In nature the bacterium causes plant tumors. [13]

Agroinfiltration experiment with *N. benthamiana*

To investigate the RNA silencing suppression activity of the coat protein (CP) and the movement protein of BMV, both genes were transiently expressed in *N. benthamiana* (separately and combined) altogether with an also transiently expressed GFP-gene. Three days after the agroinfiltration the intensity of fluorescence of the different infiltrated patches on the leaves were compared under UV-light. The GFP-gene encodes a gelly-fish (GF)-protein, which emits green fluorescence under UV-light. Naturally the GFP-gene will be partially silenced by the RNA silencing machinery of *N. benthamiana*, which leads to reduced amounts of GFP-mRNA. This in turn results in a reduced production of GFP and subsequently a reduced emission of green fluorescence. If the RNA silencing of *N. benthamiana* gets suppressed, fewer mRNAs of the GFP-gene get destroyed and therefore more GFP gets translated and the green fluorescence is significantly enhanced.

The intensity of the fluorescence of the infiltrated patches can be compared with a reference only on the same leaf. The positive or respectively negative controls, always infiltrated on the left side of the leaf, serve as an internal control. Infiltrated areas of different leaves cannot be compared with each other, due to environmental factors, such as different growth circumstances (e.g. light), which can influence the intensity of fluorescence. In this case one cannot distinguish if differences in the fluorescence intensity result from environmental effects or the actual effect of an RNA silencing suppressor, and therefore could lead to false conclusions. To overcome this problem the constructs, containing the potential RNA silencing suppressors, were infiltrated with an empty pROK vector for comparison as a negative control and on a separate leaf they were infiltrated with the 2b-gene serving as a positive control.

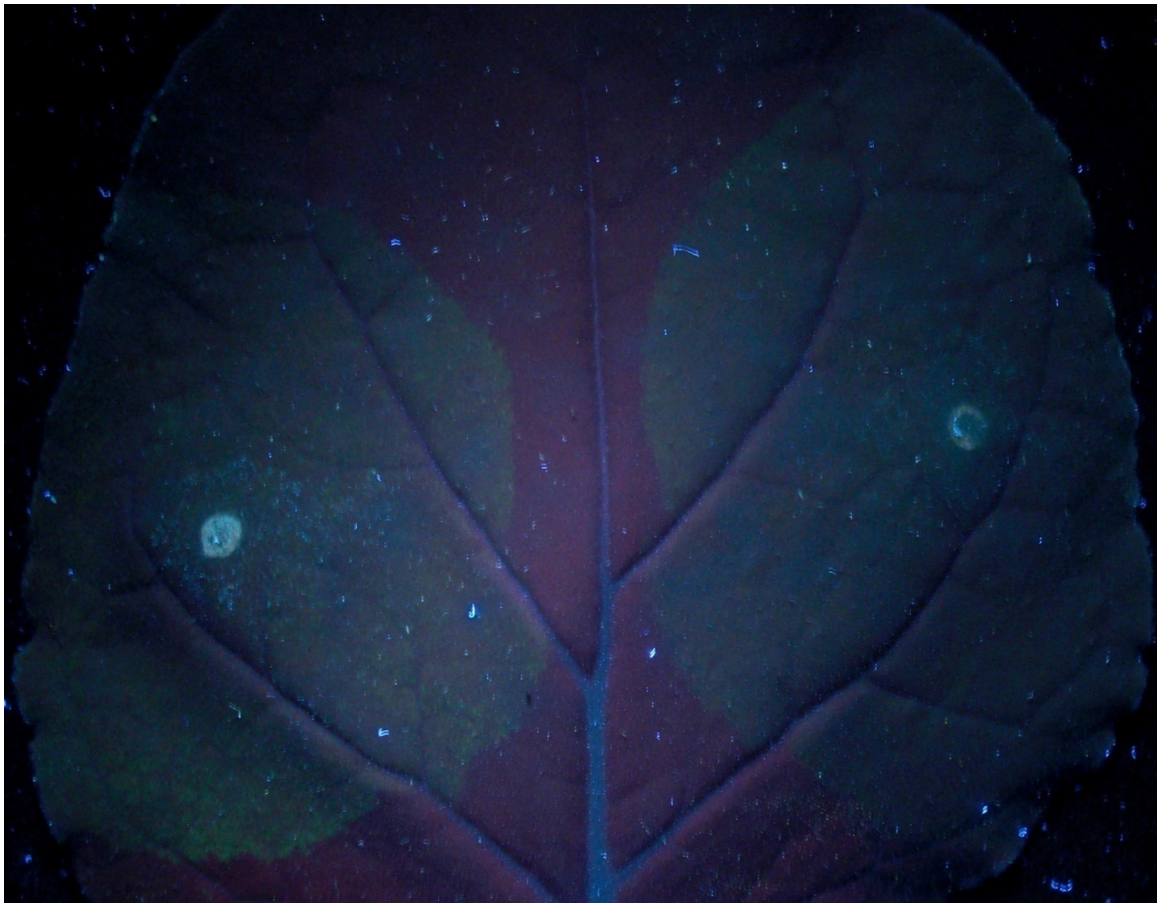

Figure [8]: On the right side the leaf was injected with a suspension of agrobacteria carrying the GFP construct. The left patch was infiltrated with a mixture of two agrobacteria suspensions, one harboring the GFP-construct and the other harboring the pROK2-construct. The GFP gene got expressed by *N. benthamiana* and subsequently partially silenced resulting in a mild fluorescence in both patches with equal intensity in most cases. Because the empty pROK2 construct doesn't express a protein, it doesn't induce RNA silencing suppression either, as expected.

Figure [8] shows the *N. benthamiana* leaf infiltrated with an empty pROK2 vector coinfiltrated with the pCambia vector carrying the GFP-gene on the left side (left patch). On the right side there was only the pCambia, GFP-gene carrying vector infiltrated (right patch). Both patches show the same intensity of green fluorescence because no RNA silencing suppressor is coinfiltrated and the GFP gene gets equally silenced.

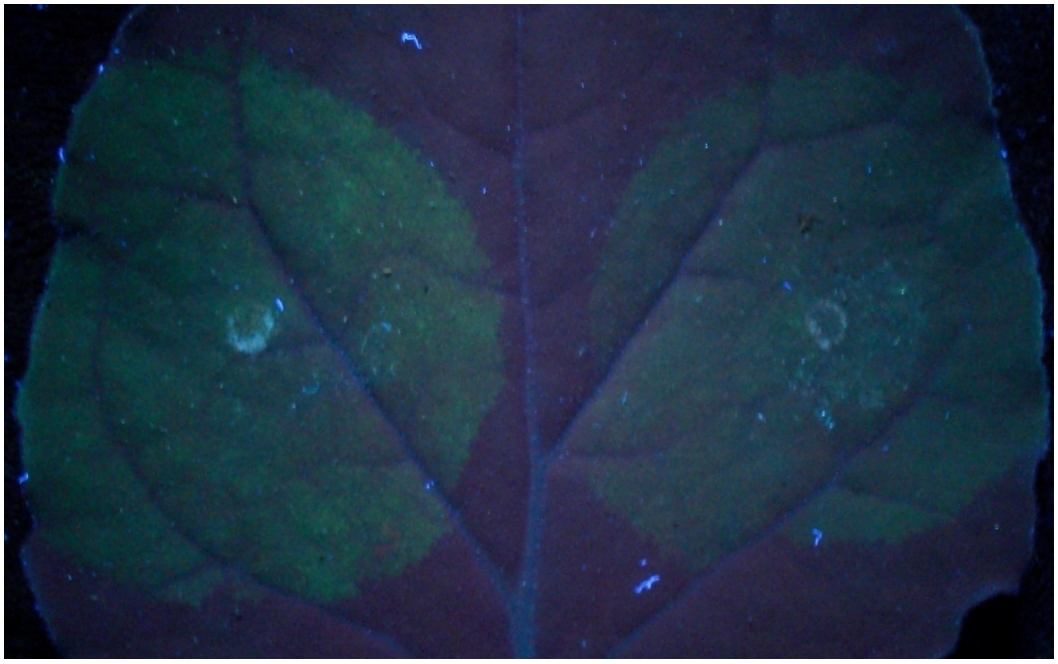

Figure [9]: On the right side, the leaf was injected with a suspension of agrobacteria carrying the GFP construct. The left patch was infiltrated with a mixture of two agrobacteria suspensions, one harboring the GFP-construct and the other harboring the pROK2-construct. The GFP gene got expressed by *N. benthamiana* and subsequently partially silenced, resulting in a mild fluorescence in both patches,

with a slightly brighter fluorescence in the left patch. Because the empty pROK2 construct doesn't express a protein, it doesn't induce RNA silencing suppression either, as expected, but it led to a little brighter fluorescence as the single GFP-construct. This effect can be explained by the fact that the RNA silencing system of the plant cells in the left patch has to deal with two different vectors, which are overcharging it and is resulting in a less efficient silencing, reflected in a marginal brighter fluorescence.

However, in some leaves the left side fluoresced slightly brighter than the right, as shown in figure [9]. This is due to the phenomenon that on the left patch the RNA silencing system has to bear two different vectors (pCambia and pROK2) and on the right patch only one (pCambia). The RNA silencing system can exclusively target only the GFP gene at the right patch, which leads to a more efficient silencing than on the left side. At the left side the silencing system has to deal with two vectors, which results in a slightly less efficient silencing and therefore brighter fluorescence.

For this reason the pCambia vector, carrying the GFP gene, was always coinfiltrated with an empty pROK2 vector and used as negative control to avoid false positive results.

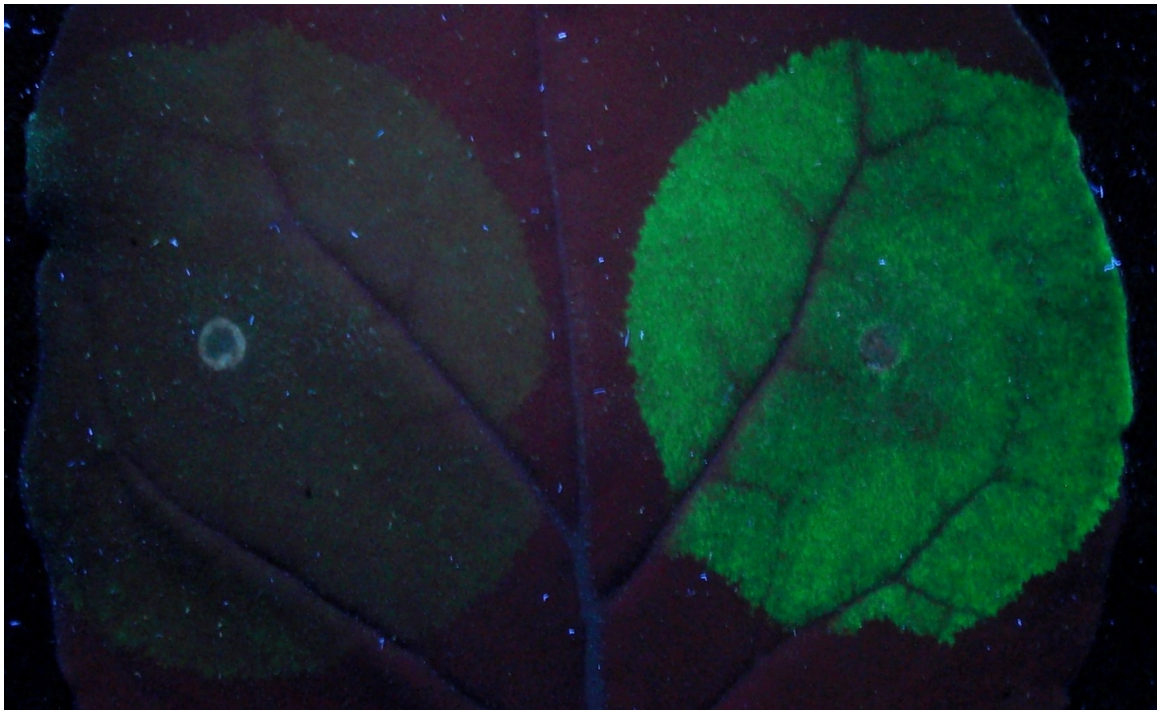

Figure [10]: On the left side, the leaf was infiltrated with a mixture of two agrobacteria suspensions, one harboring the GFP-construct and the other harboring the pROK2-construct. The right patch was coinfiltrated with the GFP-construct and the 2b-construct. The left patch emitted marginal fluorescence, because the empty pROK2 construct doesn't express a protein, and therefore it doesn't induce RNA silencing suppression of the expressed GFP gene either. The right side glowed with a very bright fluorescence, induced by the expressed 2b protein, which suppressed the silencing of the GFP gene.

To compare the negative and positive control and to proof the validity of the experiment, the negative and positive controls were agroinfiltrated next to each other on the same leaf, figure [10]. The leaf clearly shows much brighter fluorescence of the positive control on the right side caused by RNA silencing suppression of the known RNA silencing suppressor 2b.

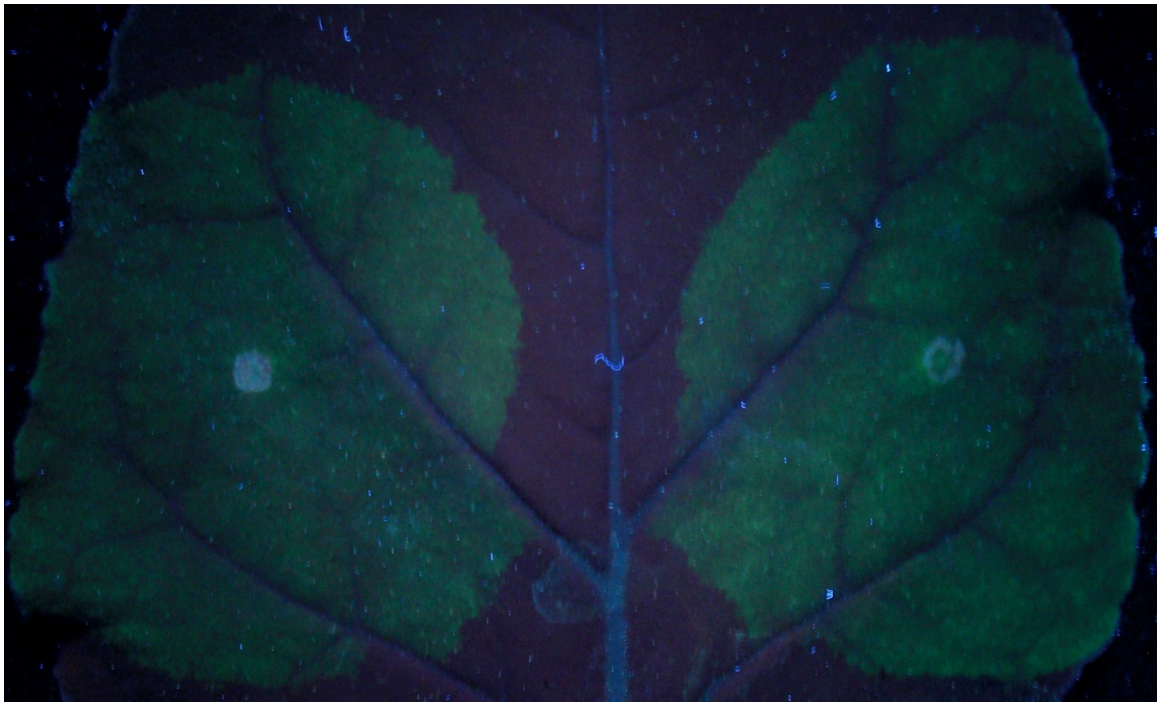

Figure [11]: On the left side, the leaf was infiltrated with a mixture of two agrobacteria suspensions, one harboring the GFP-construct and the other harboring the pROK2-construct. The right patch was coinfiltrated with GFP-construct and the

CP-construct. Both patches emitted marginal fluorescence with the same intensity, indicating that CP does not hold RNA silencing suppression activity.

To investigate the RNA silencing activity of the coat protein of BMV the pROK2 vector carrying the CP-gene was coinfiltrated with the pCambia, GFP-gene carrying construct on the right side of the leaf, figure [11]. The left side was infiltrated with the empty pROK2 vector coinfiltrated with pCambia, GFP-gene carrying vector. The left patch serves as a negative control. Both patches fluoresce with the same intensity, which clearly shows that CP does not have RNA silencing activity. In both patches the infiltrated vectors fail to induce RNA silencing activity.

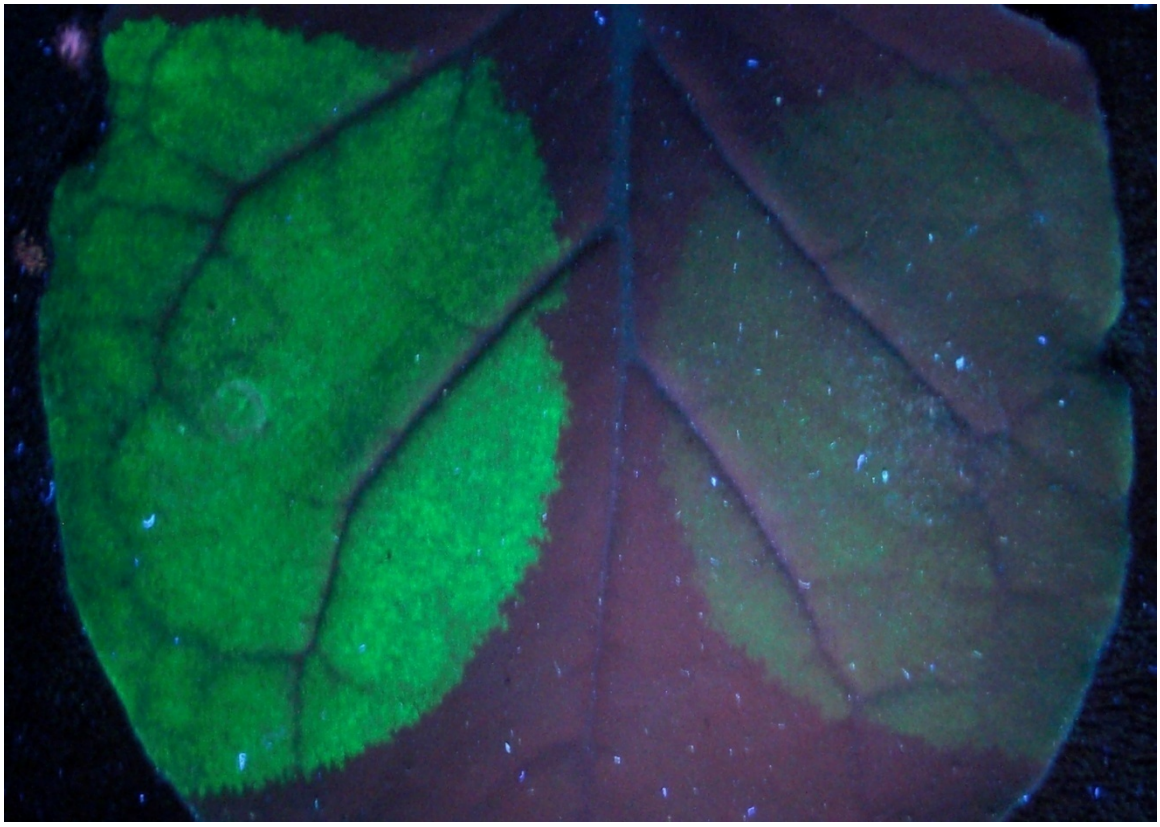

Figure [12]: On the left side, the leaf was infiltrated with a mixture of two agrobacteria suspensions, one harboring the GFP-construct and the other harboring the 2b-construct. The right patch was coinfiltrated with GFP-construct and the CP-construct. The left side glowed with a very bright fluorescence induced by the expressed 2b protein, which suppressed the silencing of the GFP gene. The right patch emitted marginal fluorescence with lesser intensity, indicating that CP does not exhibit RNA silencing suppression activity.

The comparison of the CP protein against the 2b-positive control protein confirms the lack of RNA silencing suppression activity of CP, figure [12]. The 2b protein suppresses RNA silencing very efficiently and therefore enhances the production of GFP, which creates a bright fluorescence. The CP protein, on the other hand, does not suppress RNA silencing and the GFP-gene remains suppressed, which can be observed as a very light fluorescence.

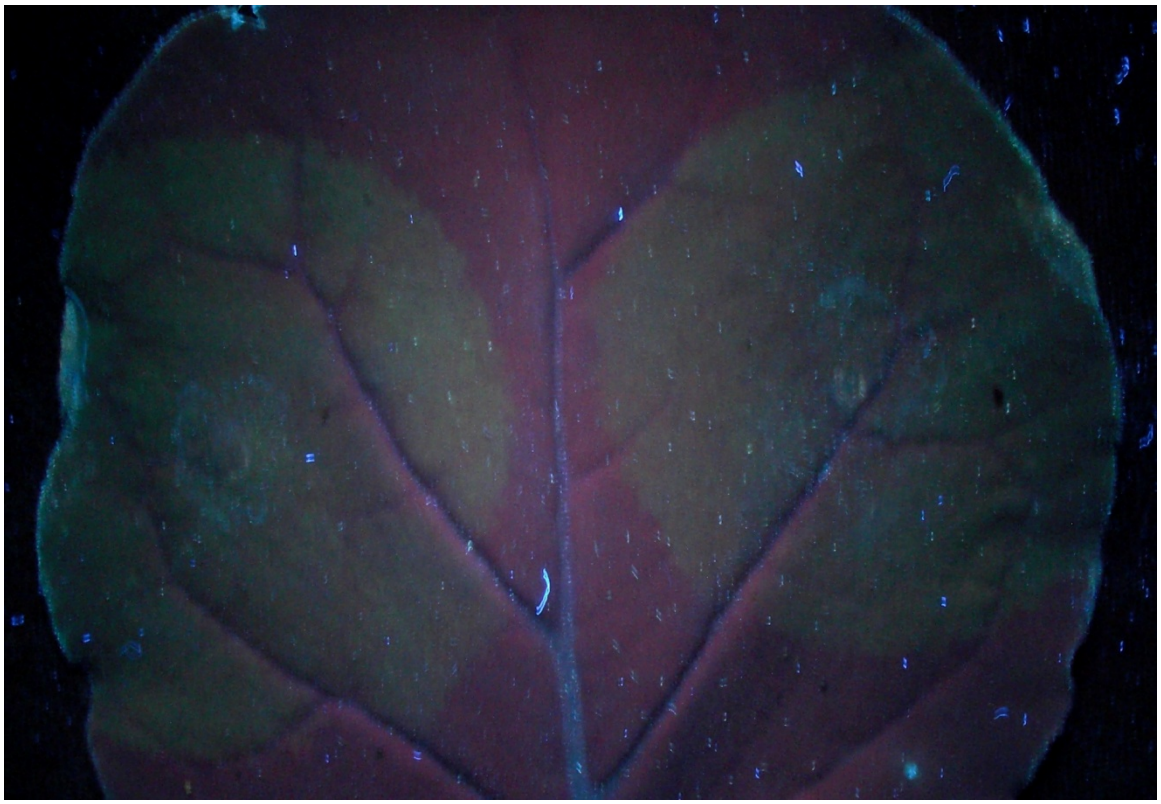

Figure [13]: On the left side, the leaf was infiltrated with a mixture of two agrobacteria suspensions, one harboring the GFP-construct and the other harboring

the pROK2-construct. The right patch was coinfiltrated with GFP-construct and the RNA3-construct. Both patches emitted marginal fluorescence with the same intensity, indicating that the movement protein does not posses RNA silencing suppression activity.

The same experiments were performed with the pROK2 vector carrying the full-length RNA3 sequence, so that only 3a movement protein was expressed. To investigate the RNA silencing activity of 3a, the pROK2 vector carrying the RNA3 sequence was coinfiltrated with the pCambia, GFP-gene carrying vector on the right side of the leaf, figure [13]. Again the left side was infiltrated with the empty pROK2 vector plus pCambia, the GFP-gene carrying vector. The left patch served as a negative control. Once more both patches fluoresce with the same intensity, clearly showing that the movement protein does not have the RNA silencing activity. Both patches fail to induce RNA silencing activity.

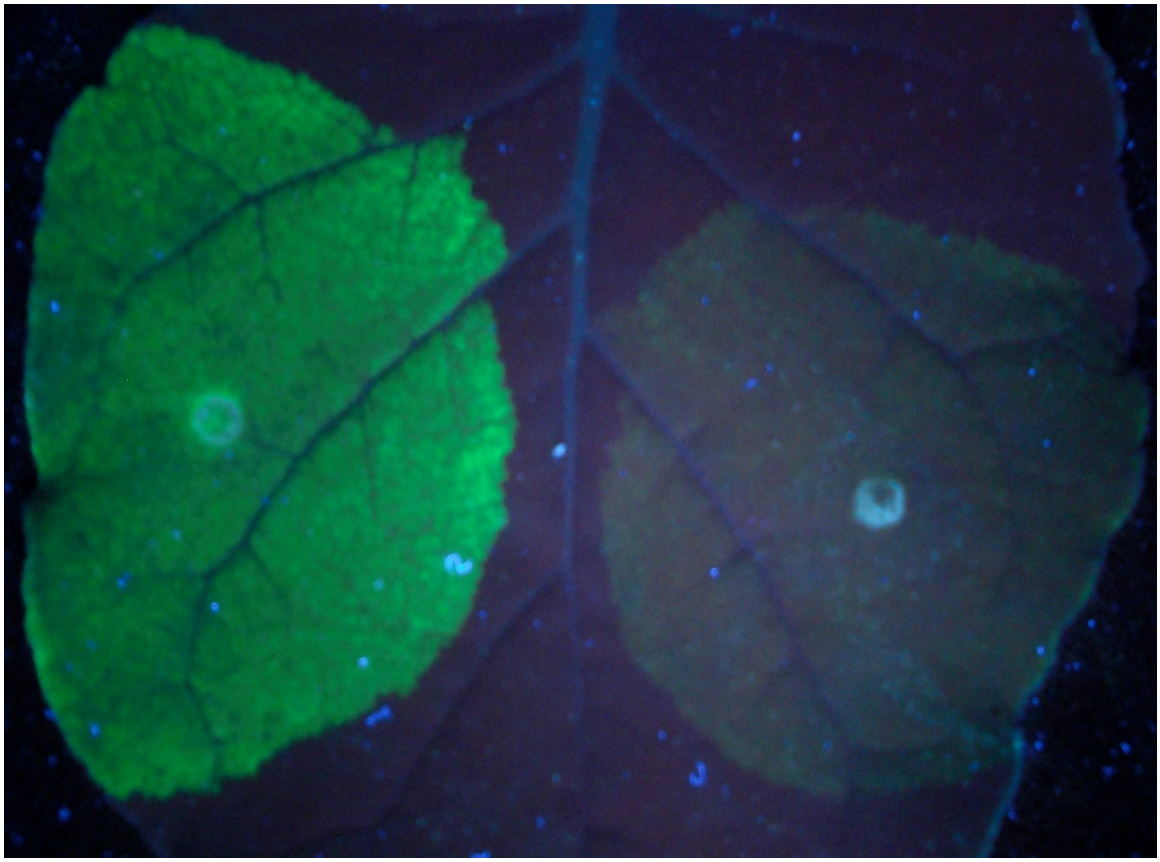

Figure [14]: On the left side, the leaf was infiltrated with a mixture of two agrobacteria suspensions, one harboring the GFP-construct and the other harboring the 2b-construct. The right patch was coinfiltrated with GFP-construct and the CP-construct. The left side glowed with a very bright fluorescence induced by the expressed 2b protein, which suppressed the silencing of the GFP gene. The right patch emitted marginal fluorescence with lesser intensity, indicating that the movement protein does not posses RNA silencing suppression activity.

A comparison against the positive control was also performed with the RNA3-construct, which verifies once more the lack of the RNA silencing suppression activity of the movement protein, figure [14]. The 2b protein suppressed RNA silencing and therefore enhanced the production of GFP, creating a bright fluorescence. The movement protein, on the other hand, did not suppress RNA silencing and the GFP-gene remained suppressed, which can be observed as a very light fluorescence.

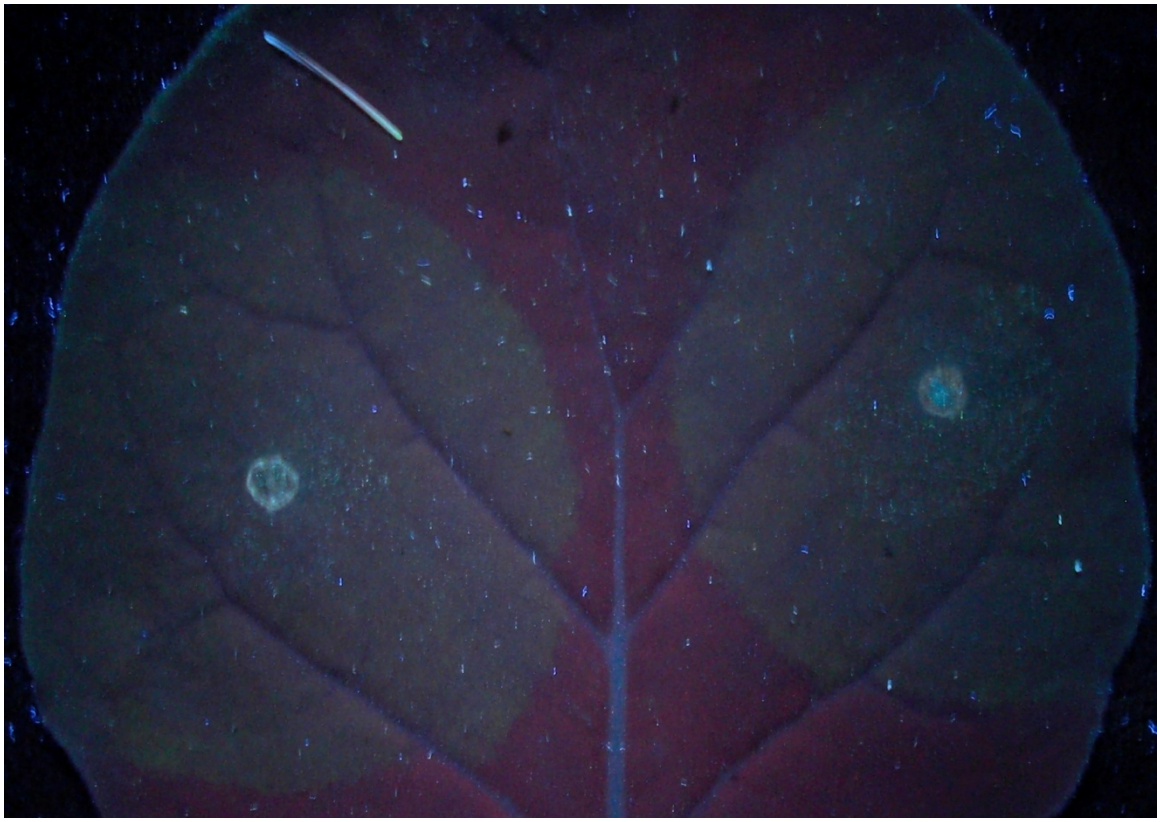

Figure [15]: On the left side, the leaf was infiltrated with a mixture of two agrobacteria suspensions, one harboring the GFP-construct and the other harboring the pROK2-construct. The right patch was triple infiltrated with GFP-construct, the RNA3-construct and the CP-construct. Both patches emitted marginal fluorescence with the same intensity, indicating that the movement protein coexpressed with CP does not possess RNA silencing suppression activity.

To exclude the possibility that only the movement protein and CP together have a RNA silencing suppression activity, both vectors, carrying one of each gene,

were coinfiltrated with the pCambia vector, carrying the GFP gene, and compared to the negative control, figure [15]. But even together these proteins did not express any RNA silencing suppression activity, proven by the same intensity of the fluorescence as a negative control.

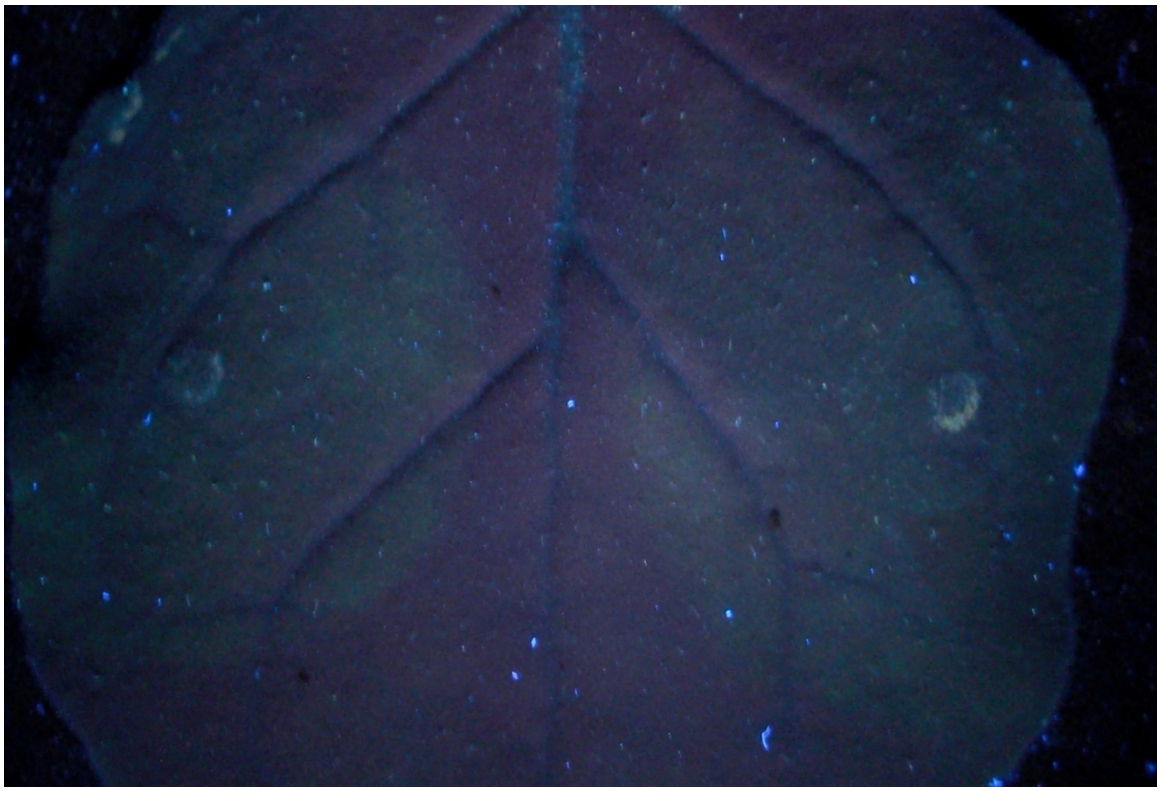

Figure [16]: On the left side, the leaf was infiltrated with a mixture of two agrobacteria suspensions, one harboring the GFP-construct and the other harboring the pROK2-construct. The right patch was coinfiltrated with GFP-construct and the RNA1-construct. Both patches emitted marginal fluorescence with the same

intensity, indicating that the 1a protein does not hold RNA silencing suppression activity.

The second last protein of BMV to inspect for RNA silencing suppression activity was the protein 1a encoded by RNA1. But as seen in figure [16], 1a does not show RNA silencing suppression activity, indicated by the same brightness of fluorescence as the negative control. The left side of the leaf was coinfiltrated with the empty pROK2 vector and pCambia, the GFP-gene carrying vector, and served as a negative control. The right side of the leaf was coinfiltrated with pROK2 carrying RNA1 and pCambia carrying GFP.

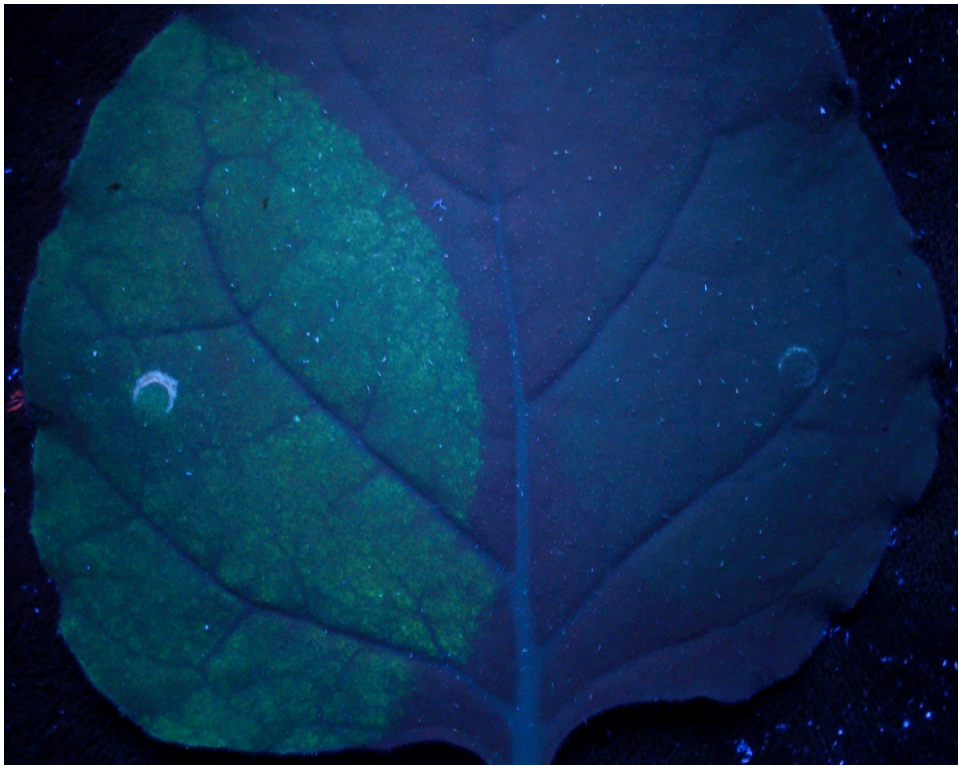

Figure [17]: On the left side, the leaf was infiltrated with a mixture of two agrobacteria suspensions, one harboring the GFP-construct and the other harboring the 2b-construct. The right patch was coinfiltrated with GFP-construct and the RNA1-construct. The left side glowed with a very bright fluorescence induced by the expressed 2b protein, which suppressed the silencing of the GFP gene. The patch on the right side emitted marginal fluorescence with lesser intensity, indicating that the 1a protein does not exhibit RNA silencing suppression activity.

To compare the 1a protein to the positive control a second leaf was infiltrated, figure [17]. The left side harbored the pROK2 vector with the 2b-gene and the pCambia vector with the GFP-gene and served as the positive control. The right side was coinfiltrated again with pROK2 carrying RNA1 and pCambia carrying GFP. The positive control showed, as expected, bright fluorescence whereas the 1a protein failed to suppress RNA silencing. The failure is indicated by the great difference in brightness between the two patches.

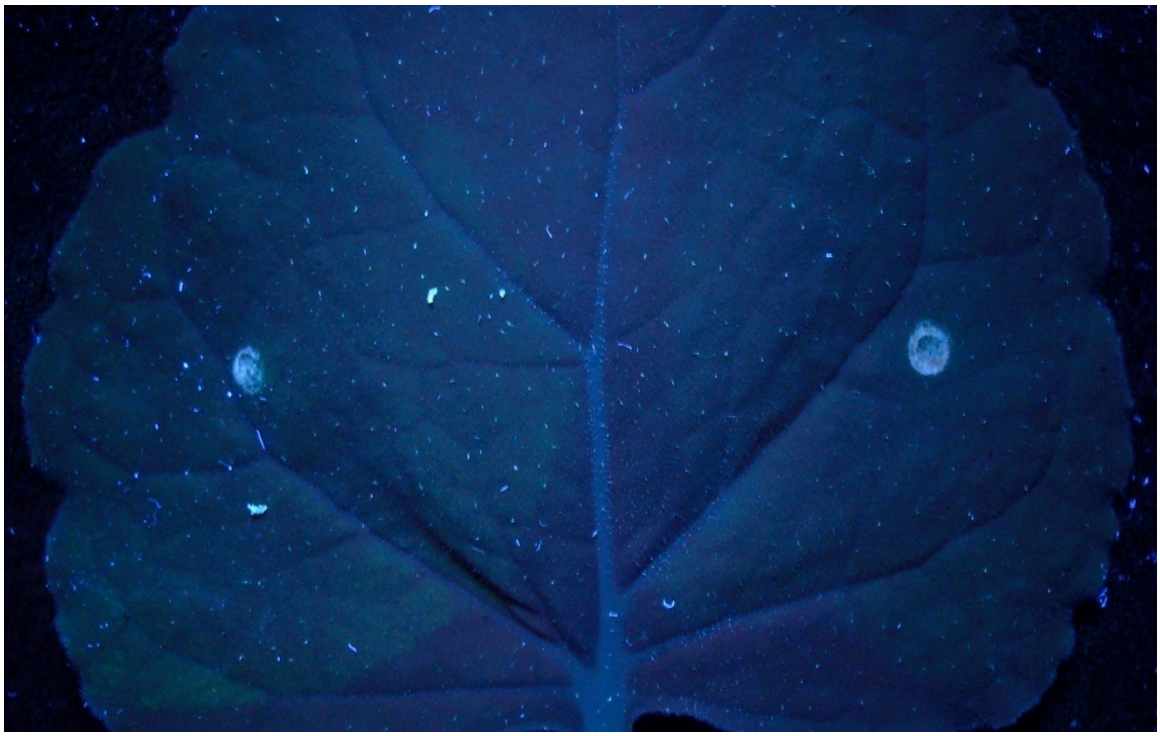

Figure [18]: On the left side, the leaf was infiltrated with a mixture of two agrobacteria suspensions, one harboring the GFP-construct and the other harboring

the pROK2-construct. The right patch was coinfiltrated with GFP-construct and the RNA2-construct. Both patches emitted marginal fluorescence with the same intensity, indicating that the 2a protein does not hold RNA silencing suppression activity.

The last protein of BMV to inspect for RNA silencing suppression activity was protein 2a encoded by RNA2. But also this protein did not show any detectable RNA silencing suppression activity, as reflected by the same brightness of fluorescence in the negative control, figure [18]. The left side of the leaf was coinfiltrated with the empty pROK2 vector and pCambia, the GFP-gene carrying vector and served as a negative control. The right side of the leaf was coinfiltrated with pROK2 carrying RNA2 and pCambia carrying GFP.

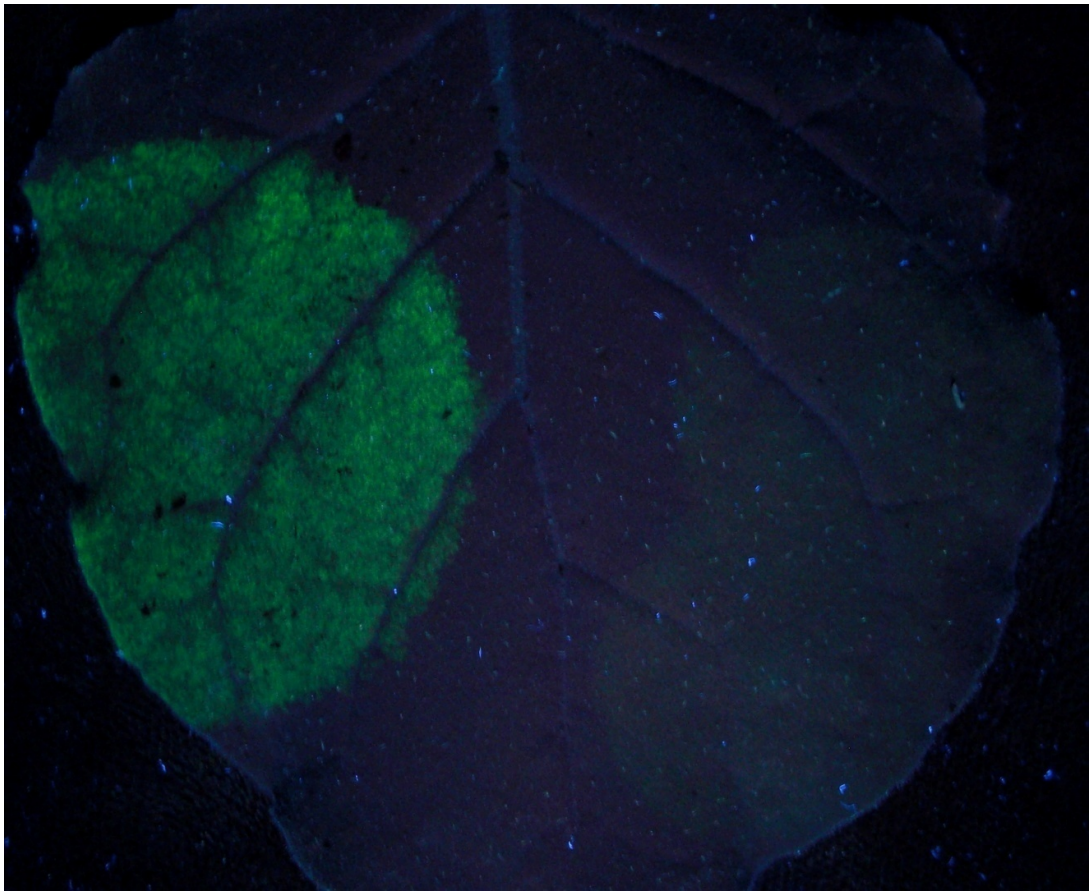

Figure [19]: On the left side, the leaf was infiltrated with a mixture of two agrobacteria suspensions, one harboring the GFP-construct and the other harboring the 2b-construct. The right patch was coinfiltrated with GFP-construct and the RNA2-construct. The left side glowed with a very bright fluorescence induced by the expressed 2b protein, which suppressed the silencing of the GFP gene. The right patch emitted marginal fluorescence with lesser intensity, indicating that the 2a protein does not hold RNA silencing suppression activity.

To compare the 2a protein to the positive control a second leaf was infiltrated, figure [19]. The left side harbored the pROK2 vector with the 2b-gene plus the pCambia vector with the GFP-gene and served as the positive control. The right side was coinfiltrated again with pROK2 carrying RNA2 and pCambia carrying GFP. The positive control showed, as expected, a bright fluorescence whereas the 2a protein failed to suppress RNA silencing. The failure is manifested by a great difference in brightness between the two patches.

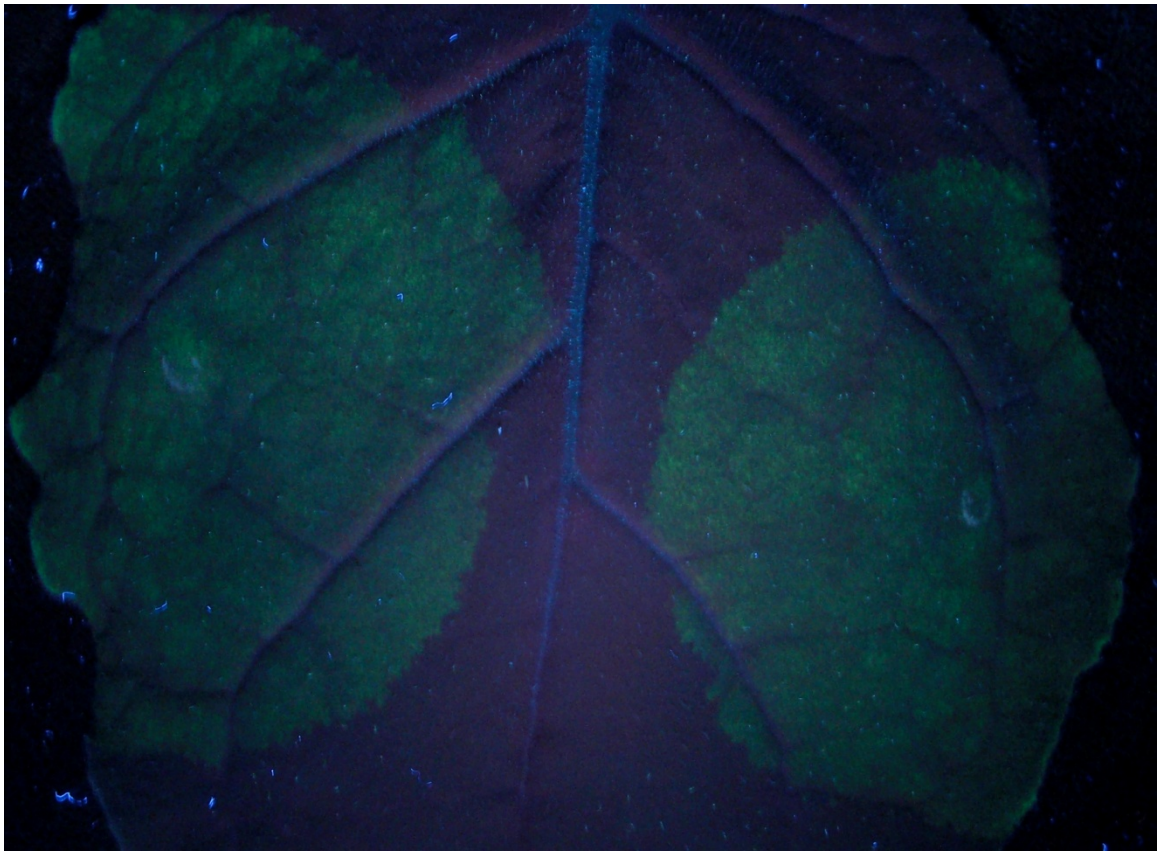

Figure [20]: On the left side, the leaf was infiltrated with a mixture of two agrobacteria suspensions, one harboring the GFP-construct and the other harboring the pROK2-construct. The right patch was triple infiltrated with GFP-construct, the RNA1-construct and the RNA2-construct. Both patches emitted marginal fluorescence with the same intensity, indicating that the 1a protein coexpressed with 2a protein does not hold RNA silencing suppression activity.

Because the 1a and 2a proteins interact to form the RNA-dependent RNA polymerase (BMV replicase) complex, both vectors carrying their genes were simultaneously infiltrated onto the leaf to check if the formed proteins function together as the RNA silencing suppressors.

But even this combined protein expression could not suppress the RNA silencing, shown in figure [20]. The left side of the leaf was coinfiltrated with an empty pROK2 vector plus pCambia, the GFP-gene carrying construct and served as a negative control. The right side was triple co-infiltrated with the GFP-construct, the RNA1-construct and the RNA2-construct. Both patches emitted very similar brightness, which demonstrates that 1a and 2a taken together do not function as the RNA silencing suppressors.

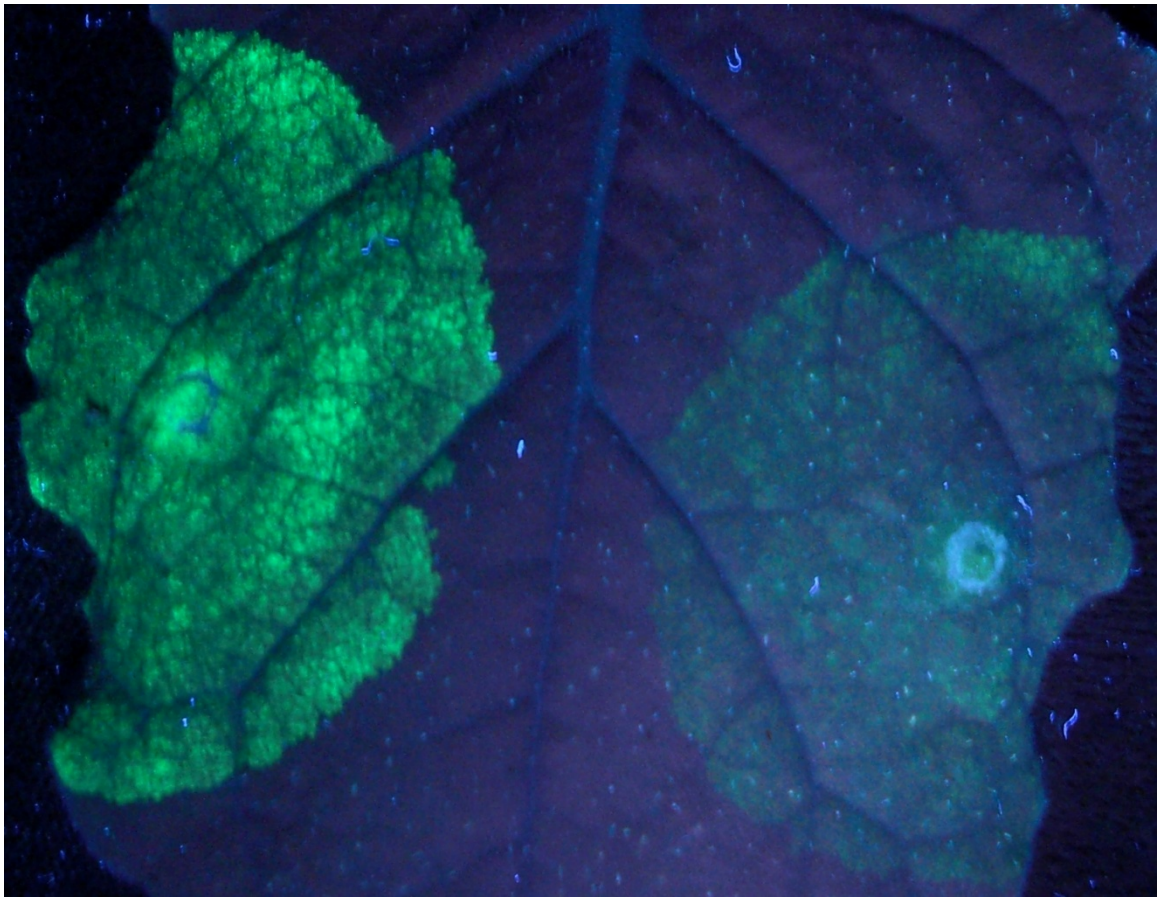

Figure [21]: On the left side, the leaf was infiltrated with a mixture of two agrobacteria suspensions, one harboring the GFP-construct and the other harboring the 2b-construct. The right patch was triple infiltrated with GFP-construct, the RNA1-construct and the RNA2-construct. The left side glowed with a very bright fluorescence induced by the expressed 2b protein, which suppressed the silencing of the GFP gene. The right patch emitted marginal fluorescence with lesser intensity,

indicating that the 1a protein coexpressed with the 2a protein does not exhibit RNA silencing suppression activity.

The combined 1a and 2a proteins were also compared to a positive control, figure [21]. The left side harbored the pROK2 vector with the 2b-gene and the pCambia vector with the GFP-gene and served as the positive control. The right side was triple co-infiltrated with pCambia carrying GFP, the RNA1-construct and the RNA2-construct. The combined proteins 1a and 2a showed again very low RNA silencing suppression activity. But the positive control demonstrated much stronger RNA silencing suppression activity, seen by the great difference in brightness.

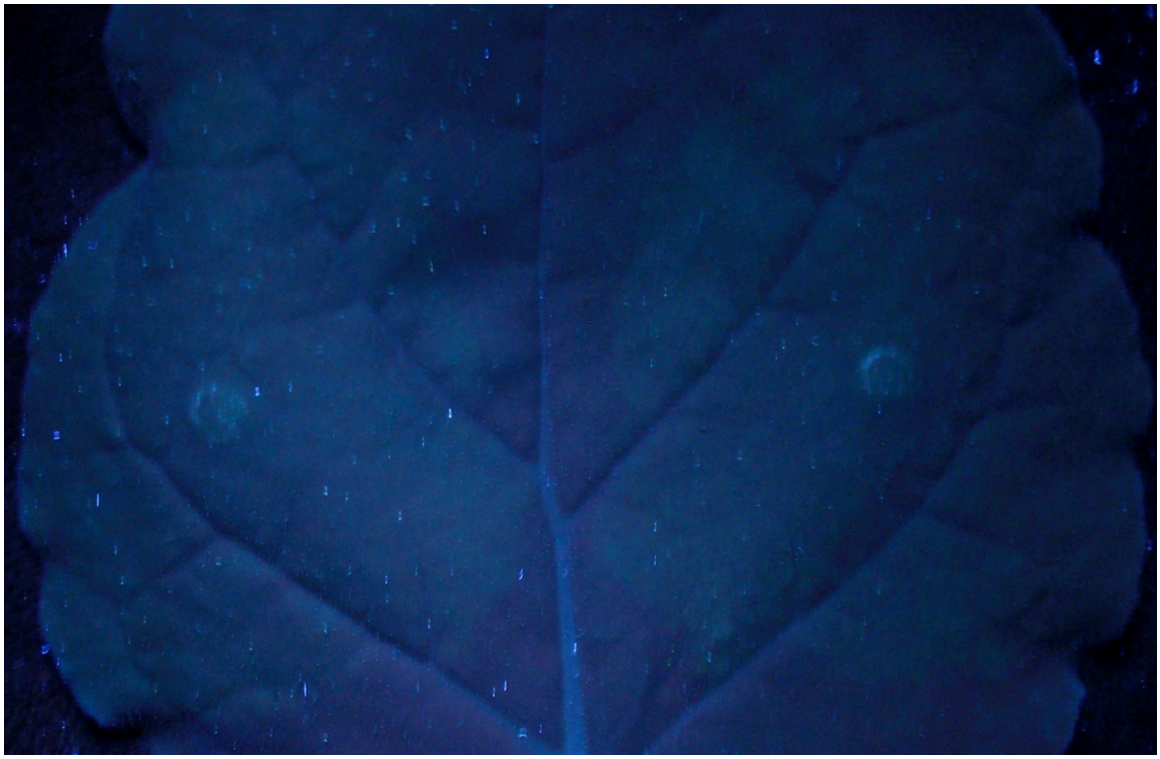

Figure [22]: On the left side, the leaf was infiltrated with a mixture of two agrobacteria suspensions, one harboring the GFP-construct and the other harboring the pROK2-construct. The right patch was quadruple infiltrated with GFP-construct, the RNA1-construct, the RNA2-construct and the RNA3-construct. Both patches emitted marginal fluorescence with the same intensity, indicating that the coinfiltrated proteins 1a, 2a and 3a do not hold RNA silencing suppression activity.

All three RNAs had been quadruple co-infiltrated altogether to draw a final conclusion, figure [22]. The left side of the leaf was coinfiltrated with the empty

pROK2 vector and pCambia, the GFP-gene carrying construct and served as a negative control. And the right side was co-infiltrated with the GFP-construct, together with the RNA1- RNA2-and RNA3-constructs. Both patches emitted the same brightness, which shows that 1a, 2a, and 3a do not have RNA silencing suppression activity together.

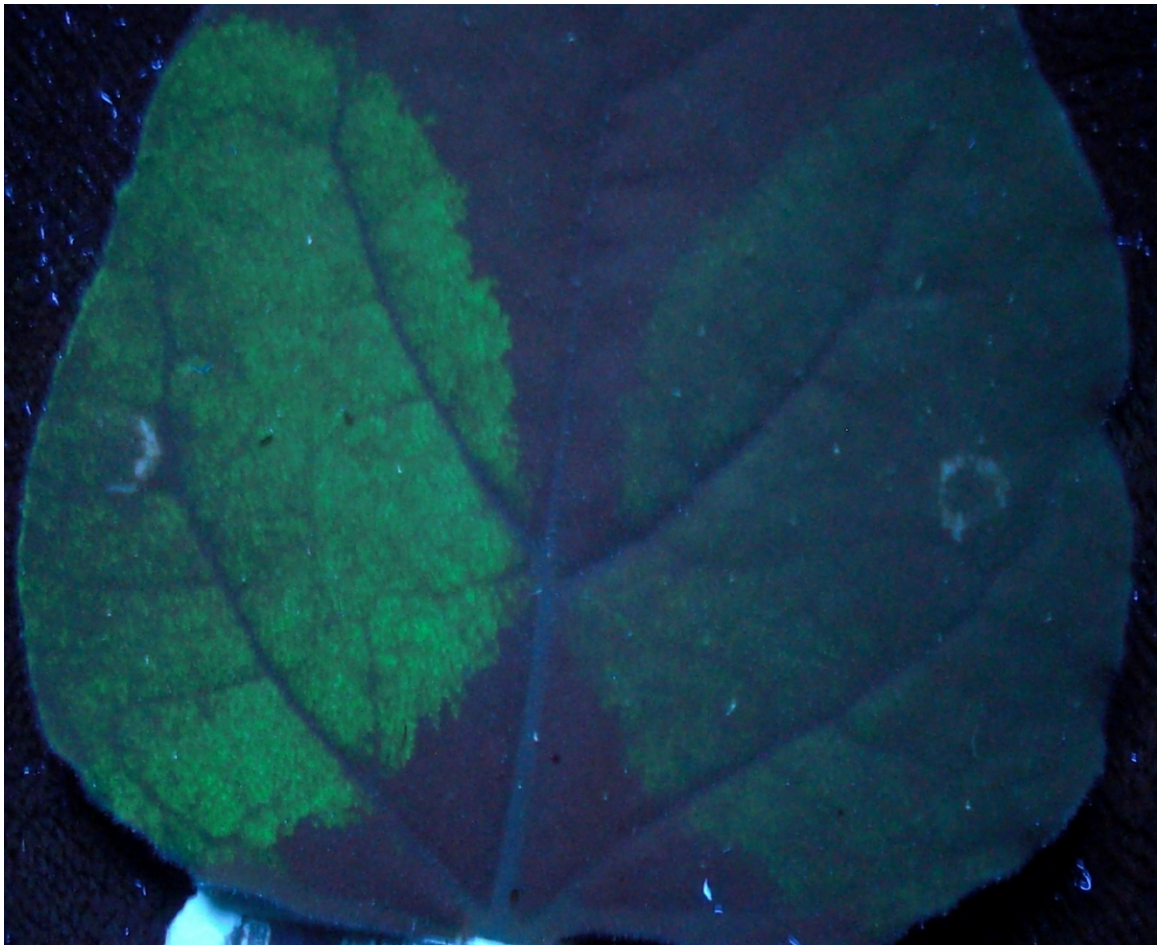

Figure [23]: On the left side, the leaf was infiltrated with a mixture of two agrobacteria suspensions, one harboring the GFP-construct and the other harboring the 2b-construct. The right patch was quadruple infiltrated with GFP-construct, the RNA1-construct, the RNA2-construct and the RNA3-construct. The left side glowed with a very bright fluorescence induced by the expressed 2b protein, which suppressed the silencing of the GFP gene. The right patch emitted marginal

fluorescence with lesser intensity, indicating that the coinfiltrated proteins 1a, 2a and 3a do not exhibit RNA silencing suppression activity.

The combined 1a, 2a and 3a proteins were also compared to the positive control, figure [23]. The left side harbored the pROK2 vector with the 2b-gene and the pCambia vector with the GFP-gene and served as the positive control. The right side was infiltrated with pCambia carrying GFP, the RNA1-, RNA2- and RNA3 constructs. The combined proteins 1a, 2a and 3a displayed again no RNA silencing suppression activity. In contrast, the positive control demonstrated a strong RNA silencing suppression activity, as seen by the great difference in brightness.

Agroinfiltration experiment with Chenopodium quinoa

The agroinfiltration experiment was also performed in the same manner with a different host, Chenopodium quinoa. Chenopodium quinoa turned out to be an inadequate host for this experiment. A stronger force was needed to inject the agrobacterium suspension into the leaf, the syringe had to be applied several times and the suspension did not spread much further beyond the point of application. Although the leaf showed very light fluorescence, just from the bottom side of the leaf, it was impossible to distinguish between the fluorescence among the patches.

The fluorescence was highly diffused and it could not be used for the experiment, because it failed to give any information about the different intensities between negative and positive controls. One leaf is shown in figure [24] as an example of a high number of the injected Chenopodium quinoa leaves. None of them showed any difference in the fluorescence regardless of the infiltrated constructs.

I conclude that Chenopodium quinoa cannot be used for the agroinfiltration experiments.

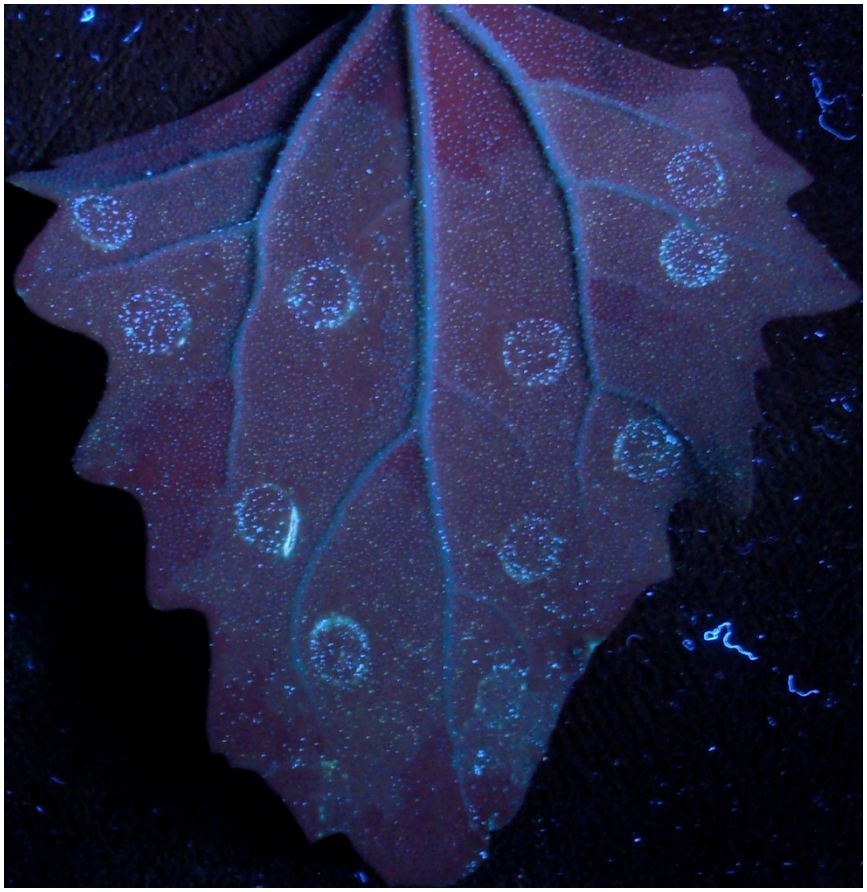

Figure [24]: *Chenopodium quinoa*. On the left side, the leaf was infiltrated with a mixture of two agrobacteria suspensions, one harboring the GFP-construct and the other harboring the 2b-construct. The right patch was coinfiltrated with the GFP-construct and the RNA3-construct. Both sides emitted marginal, diffuse fluorescence. The 2b protein failed to suppress RNA silencing in this host, which makes *Chenopodium quinoa* insufficient for the agrointiltration experiment.

Agroinfiltration experiment with barley leaves (*Hordeum vulgare)*

A second attempt to use a different host for the agroinfiltration experiment was conducted. Only barley plants in the single leaf stadium were used for this experiment. The fully expanded leaves were treated in two different ways. First, the leaves were tested the same way as described before, by injecting the agrobacteria suspension. In another approach, the leaves were cut and incubated standing in the agrobacterium suspension for 3 days. The agrobacterium mixture was made of 2 different agrobacteria suspensions. The first part of the 2-component mixture consisted of the agrobacterium harboring the GFP construct in all cases; the second component included an agrobacterium harboring the pROK2 construct (negative control), the 2b construct (positive control), the RNA3-construct or the CP construct. The idea was that the leaves would take up the suspension and express the genes. Barley is another natural host for BMV and it was intended to confirm the results already obtained on *N. benthamiana*.

Figure [25]: *Hordeum vulgare* was incubated in a mixture of two agrobacteria suspensions, one harboring the GFP-construct and the other harboring the 2b-construct. *Hordeum vulgare* emitted no fluorescence. The lack of fluorescence makes *Hordeum vulgare* insufficient for the agrointiltration experiment.

The barley leaves incubated standing in the agrobacterium mixtures, figure [25], failed to produce fluorescence in any combination of suspensions. Neither the positive control, which had been barley leaves incubated in an agrobacterium mixture harboring the GFP construct and the 2b construct, nor any other leaves showed fluorescence. The absence of fluorescence indicates that the GFP construct, which was included in every suspension, was not present due to the inability of the leaves to take up the suspension or due to a failed expression. In any case this proves that this experiment is unusable for transient expression experiments.

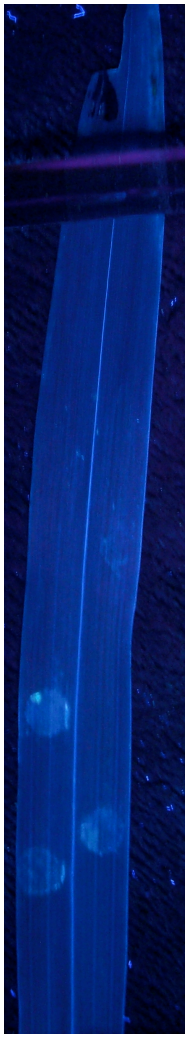

Figure [26]: *Hordeum vulgare*. On the left side, the leaf was infiltrated with a mixture of two agrobacteria suspensions, one harboring the GFP-construct and the other harboring the pROK2-construct. The right side was coinfiltrated with the GFP-construct and the 2b-construct. Both sides emitted almost no fluorescence. The observed fluorescence is too little for an evaluation, which makes *Hordeum vulgare* insufficient for the agroinfiltration experiment.

The barley leaves, infiltrated with the agrobacterium suspension in a similar manner as described before, failed to produce fluorescence as well, figure [26]. The barley leaves had been injected with an agrobacterium mixture that was made of 2 different agrobacteria suspensions. One side of the leaf was infiltrated with the negative control, pROK2 construct coinfiltrated with the GFP construct, or the positive control, GFP construct coinfiltrated with the 2b construct. The other side was injected with the GFP construct coinfiltrated with the RNA3 construct or the CP construct. None of the leaves, treated in this manner, emitted useable fluorescence. In some leaves very small patches with very light fluorescence could be found, as seen in figure [26] in the upper right side. But these patches occurred randomly and were not usable for the agroinfiltration experiment. In this experiment the disability of uptake could be excluded, by direct infiltration, but sill the expression of the vectors fails. The three circular dots on the leaf in the figure [26] are caused by the impression of the tip of the syringe during the infiltration process and do not represent fluorescence. In conclusion, barley cannot be used for the agroinfiltration experiments.

Protein verification

For a final proof that the constructs were expressed and that the proteins were actually present in the leaf tissue, WESTERN blot analysis were performed. Since both proteins of the RNA3 component failed to suppress RNA silencing, it was necessary to show that this was not the absence of the proteins as a reason for the lack of suppression but their inner inability per se to function as the RNA silencing suppressors.

Movement Protein WESTERN Blot

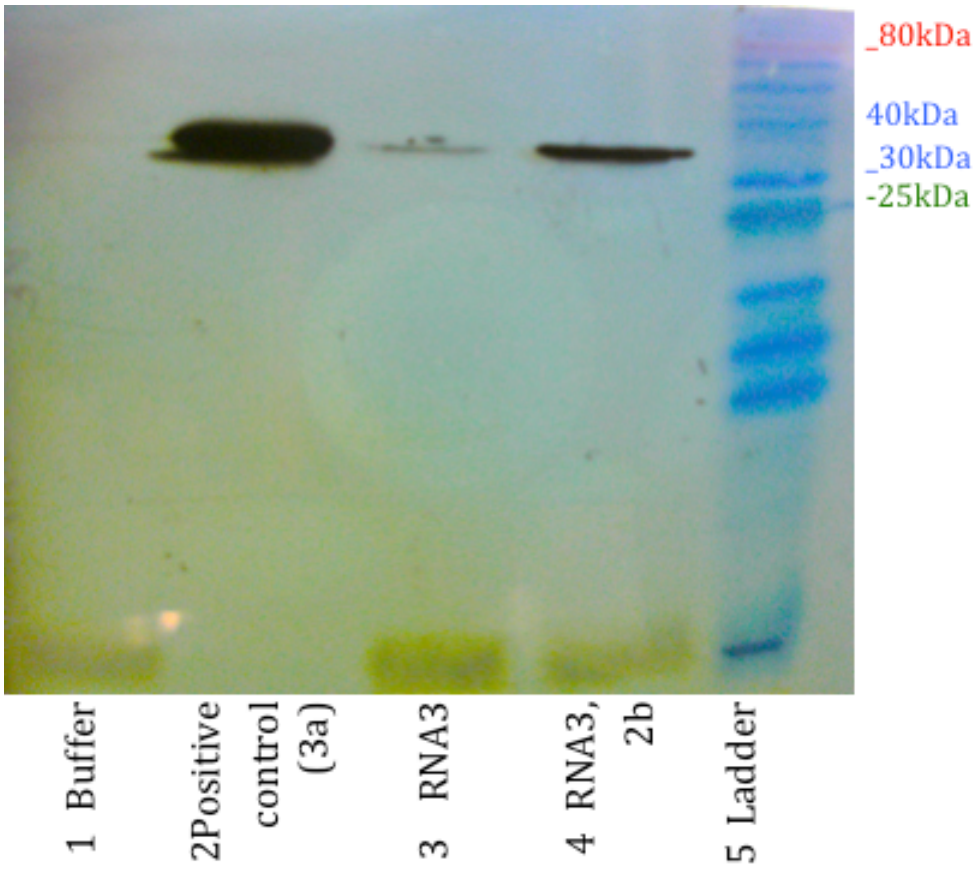

Figure [27]: WESTERN blot of agroinfiltrated leaves, confirmation of movement protein expression in agroinfiltrated tissue. Lane 1 was loaded with total protein extraction of a leaf infiltrated with buffer as a negative control and does not show a band, since no movement protein was expressed. Lane 2 shows the positive control, the movement protein obtained from an in vitro, wheat germ translation kit. The band in lane 3 exhibits the movement protein of the total protein extraction of a leaf infiltrated with the RNA3 construct. This lane appears at the size of the movement

protein proven by the positive control and the ladder. Lane 4 exhibits the lane of the total protein extraction of a leaf infiltrated with the constructs RNA3 and 2b. The band proves the expression of the movement protein as well. In lane 5 a protein ladder migrated.

A total protein extraction of agroinfiltrated *N. benthamiana* leaves was performed to confirm the presence of the movement protein, figure [27]. One leaf of *N. benthamiana* was infiltrated only with the buffer and served as a negative control. A second leaf was infiltrated with the pROK2 construct carrying the entire BMV RNA3 sequence. And a third leaf was co-infiltrated with the pROK2 vector carrying BMV RNA3 plus the pROK2 vector carrying the CMV 2b-gene.

As expected, the buffer-infiltrated leaf does not show a band for the movement protein as depicted in lane 1. The leaf agroinfiltrated with RNA3 shows a band of the size of the movement protein (32kDa), lane 3. This band also comigrated with the positive control, lane 2. As positive control the purified movement protein was run on the gel. The positive control was obtained from the co-worker Michael Flinn who, used a MEGAscript® T7 High Yield Transcription Kit from Ambion to transcribe a RNA3 construct as instructed in the manual. The RNA was subsequently translated using a Wheat Germ Extract *In Vitro* Translation Kit purchased from Promega. Lane 4 depicts the protein extracted from the leaf coinfiltrated with the

pROK2 vector carrying the total RNA3 plus the pROK2 construct carrying the 2b-gene. This lane also confirms the presence of the movement protein, as the band appears at the right size (32kDa) and at the same height as the positive control. Apparently, more 3a protein accumulated in the tissue, likely due to the RNAi suppressing activity of protein 2b. Furthermore, the bands confirm the presence of the movement protein itself, as they are the product of the binding of a specific antibody to the movement protein. In lane 5 a protein ladder is pictured which serves to confirm the correct size of the movement protein (32kDa). The band of the movement protein in lane four is markedly more intensive than the band in lane three although the same amount of the leaf tissue was used for protein extraction. This additionally confirms that the co-infiltrated 2b-gene successfully suppressed the RNA silencing. Furthermore, this WESTERN blot shows that the construct carrying the full-length RNA3 sequence is able to express the movement protein in *N. benthamiana*, but the movement protein is unable to suppress RNA silencing.

Coat Protein WESTERN Blot

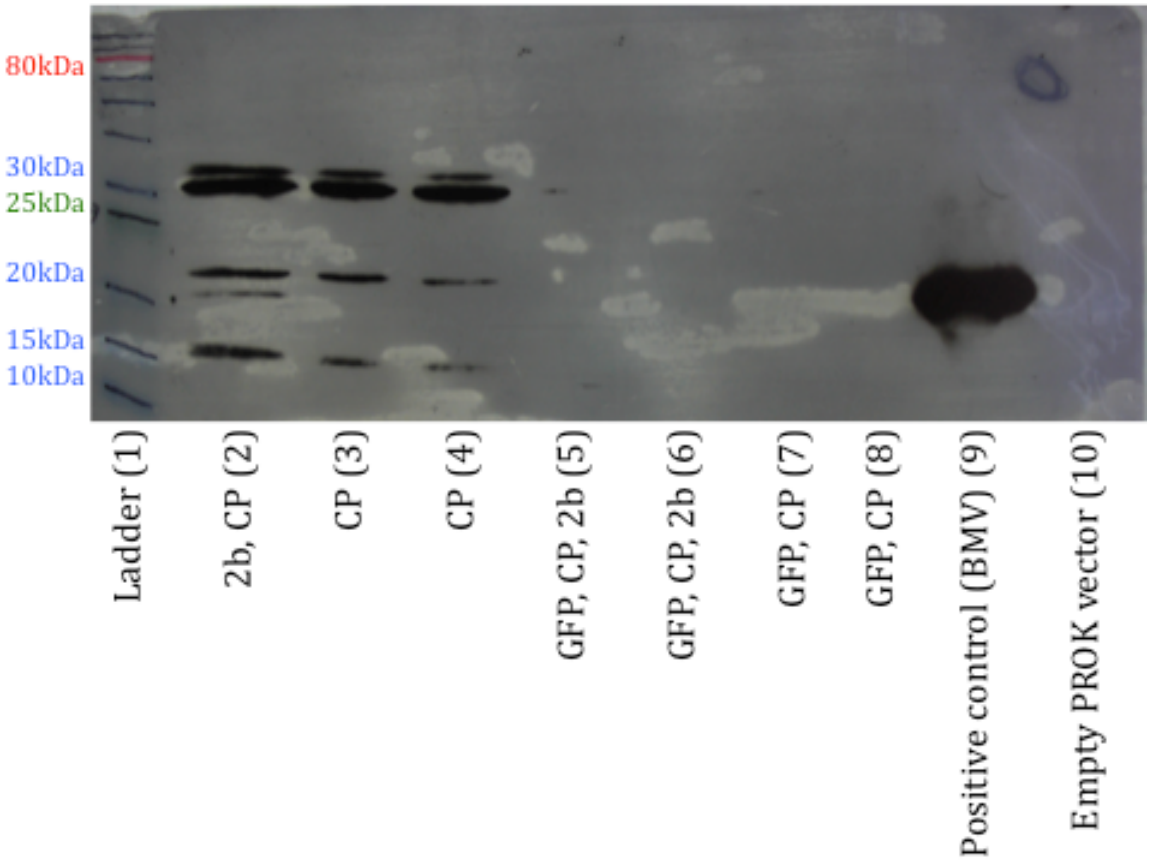

Figure [28]: WESTERN blot of agroinfiltrated leaves, confirmation of CP expression. In lane 1 a protein ladder migrated. Lane 2 was loaded with a total protein extraction of a leaf infiltrated with the 2b- and CP-construct. The lane exhibits 5 bands, one at the expected size of 21 kDa for CP, as can be compared with the ladder and the positive control, which proves the expression of CP in *N. benthamiana*. The other bands result from unspecific binding, aggregation or degradation of CP. The same combination of bands is expressed in lanes 2 and 3 with a lesser intensity due

to the lack of a RNA silencing suppressor. These lanes were loaded with the total protein extraction of leaves infiltrated with only the CP-construct, which verifies the expression of the construct. Lanes 5, 6, 7, 8 and 10 do not show bands due to failed protein extraction. Lane 9 was loaded with BMV itself and served as a positive control for CP.

A total protein extraction of the agroinfiltrated *N. benthamiana* leaves was performed to confirm the expression of the coat protein (CP), figure [28]. Leaves of *N. benthamiana* were co-infiltrated with the CP-construct alone, the CP-construct together with the 2b-construct, the CP-construct plus GFP-construct, the CP-, GFP-, and the 2b-constructs or only with the empty pROK2 vector. Prior attempt of the total protein extraction failed due to the extraction buffer, which needed to be optimized. For that reason lanes 5-8 and 10 do not show any proteins.

Lane nine represents the positive control, which is the BMV virus preparation itself. A band at about 20 kDa is visible, the size of the coat protein. Furthermore, in lane one a protein ladder was run to confirm the size of the coat protein. Its 20 kDa band migrated the same distance as the positive control, proving the CP by its size. In lanes three and four the total protein extracts from leaves infiltrated with the CP-construct alone were run. Both lanes show bands at about 20kDa that comigrate with the BMV CP standard (lane 8), proving the expression of

CP. The bands above and below the coat protein band could be generated by aggregation or degradation of the coat protein, respectively. The same composition of bands appears at lane two of a total protein extract from the leaf coinfiltrated with the CP- and 2b-constructs, but with a slightly higher intensity. This reveals once again the RNA silencing suppression activity of the 2b protein, which results in a higher CP concentration in the infiltrated tissue.

In conclusion, this WESTERN blot analysis confirms that the construct carrying the CP-ORF is able to express the coat protein in *N. benthamiana*, but that the coat protein also fails to induce a detectable RNA silencing suppression.

Chapter 4

Discussion

Agroinfiltration experiment

The goal of this study was to determine if the proteins encoded by BMV, a plant RNA virus, display any RNA silencing suppression activity. For this purpose agroinfiltration of *N. benthamiana* plants carrying different Ti-based constructs with various BMV genes silence the RNAi activity in *N. benthamiana*, respective to GFP, a reporter gene. The transient expression vectors are widely used to test the RNA silencing suppression activity in the molecular biology research. The construct containing the GFP gene induces RNA silencing, whereas the other constructs will suppress the silencing, if indeed they function as the silencing suppressors [3;8].

The validity of this experiment is proven by a direct comparison with both the negative and positive controls, figure [10]. A leaf infiltrated with the empty pROK2 vector and the vector carrying the GFP-gene on the left side of the leaf and the 2b-gene- and the GFP-gene-constructs on the right side of the leaf are shown. The left side of the leaf displays mild fluorescence due to the RNA silencing activity of *N. benthamiana* since the empty pROK2 vector carries no RNA silencing suppressor (negative control). The right side of the leaf shows significantly higher

fluorescence due to the suppression activity of the 2b-gene, a well-known RNA silencing suppressor (positive control). The result clearly depicts the difference of the intensity of fluorescence between the positive and the negative controls.
All proteins encoded by BMV show a clear outcome from these experiments, because all of them display a similar fluorescence to the negative control. (Figure [11] [13] [15] [16][18][20][22]). This clearly demonstrates that neither the proteins encoded by RNA 1 and RNA2 nor the coat protein nor the movement proteins possess the RNA silencing suppression activity. These results are backed up once more by the comparison of the intensity of fluorescence of the proteins to be tested to the proteins expressed by a construct serving as positive control (a well-known RNA silencing suppressor of CMV: 2b protein) (Figure [12] [14][17][19][21][23]). These results first of all show that the experiment works and that the RNA silencing suppression can markedly increase the fluorescence. Secondly, none of the BMV proteins does enhance the intensity of fluorescence. In all examined 150 leaves that were infiltrated during the course of repeated experiments, the positive and negative control always showed an unchanged intensity of fluorescence and the proteins tested for RNA silencing suppression never gave a different result, which reflects the excellent reproducibility and the high specificity of such experiments.

Protein verification

Because neither the coat nor the movement proteins have shown a detectable RNA silencing suppression activity, it was important to prove that the particular proteins were actually expressed in *N. benthamiana*. This then demonstrated that insufficient transcription or translation were not the reason for the lack of suppression activity. The performed WESTERN blots indeed confirmed that both proteins were expressed in the infiltrated plant tissue. The presence of both CP and 3a proteins were verified by their size, by the comparison to positive controls, and by the specificity of particular probing antibodies.

Both experiments together ultimately show the lack of detectable RNA silencing suppression activity for all four BMV-encoded proteins. However, the expression of 1a and 2a proteins remains to be demonstrated in the infiltrated patches. On the other hand, we believe this is not sine qua non necessary because these two particular constructs were a generous gift of Dr. CC Kao and they have already shown their expression activity in *N. benthamiana* tissue [12].

The fact that none of the BMV proteins have RNA silencing suppression activity according to the presented experiments stands in great contradiction to the general presumption that most plant viruses encode for at least one RNA silencing

suppressor [14]. Furthermore, many papers even indicate that every plant virus has to have one or more RNA silencing suppressor(s) [15]. In fact, RNA silencing suppressors are already known for many plant virus species [16].

But not all RNA silencing suppressors are equally efficient. Some have weaker RNA silencing suppression activity. For example p25 of *Potato virus X* (PVX) has a lower RNA silencing suppression activity, compared to the CP of the *Turnip crinkle virus* (TCV), which has a strong suppression activity in the agroinfiltration experiment [17]. The knowledge of the gradual activity of identified RNA silencing suppressors could lead to the hypothesis that BMV could have an RNA silencing suppressor that has very weak suppression activity, too weak to be detected with the agroinfiltration assay.

Another explanation for the negative result of the agroinfiltration experiment might be that BMV does not have a local RNA silencing suppressor, but a systemic suppressor. The agroinfiltration assay cannot identify systemic RNA silencing suppressors. Systemic silencing is determined by systemic silencing signals which travel through the vascular system to remote parts of the plant. Short double stranded RNAs, its precursors and nucleic acids in general are assumed to make up the systemic and local RNA silencing signals [18]. It could be possible that BMV possesses a RNA silencing suppressor that only suppresses systemic silencing.

Other known examples of systemic silencing suppressors are: the suppressor HC-Pro of potato virus Y (PVY) and P1 of rice yellow mottle virus (RYMV). They cause systemic RNA silencing suppression by specially blocking 24-nucleotide-long short RNAs. Other suppressors that block 21- and 24-nucleotide-long short RNAs affect both local and systemic RNA silencing suppression, like the P19 protein of tomato bushy stunt virus (TBSV) [19]. The coat protein of *Citrus tristeza virus* (CTV) is another example of a RNA silencing suppressor that only possesses systemic activity. It cannot be detected by the agroinfiltration experiment, but with a special assay for systemic RNA silencing suppression [4;20]. This could also be the case for BMV and would explain the absence of suppression activity in the agroinfiltration experiment.

Chapter 5

Future Research

Although the agroinfiltration experiment clearly shows that none of the proteins of BMV display a strong RNA silencing suppression activity, it could be that one or more of these BMV protein display so-called systemic RNA silencing activity. The agroinfiltration experiments used in this work have allowed us to determine only a local RNA silencing suppression.

Some already characterized RNA silencing suppressors do exhibit systemic RNA silencing activity, including the coat protein of *Citrus tristeza virus* (CTV). This type of suppressors can only be detected with another experiment, which involves grafting of transgenic plants which express the putative RNA silencing suppressor. Such a plant has to be crossed with another transgenic plant that carries an autonomously silencing reporter gene. This assay would have to be performed in order to give the final conclusion of the RNA silencing suppression activity of BMV proteins [4]. Yet another aspect of RNA silencing suppression is the quantitative feature. That is, that some viruses require only a low level of suppression whereas more aggressive viruses usually suppress the RNA interference more efficiently. These and similar questions will be the subject of our future experiments in order to find out how bromoviruses stay away from the host RNAi surveillance.

References

[1]

C. CHENG KAO AND K. SIVAKUMARAN. Pathogen profile Brome mosaic virus, good for an RNA virologist's basic needs. MOLECULAR PLANT PATHOLOGY (2000) 1(2), 91–97 Blackwell Science, Ltd

[2]

Lisa K. Johansen and James C. Carrington. Silencing on the Spot. Induction and Suppression of RNA Silencing in the Agrobacterium-Mediated Transient Expression System1. Plant Physiology, July 2001, Vol. 126, pp. 930–938.

[3]

Virginia Ruiz-Ferrer and Olivier Voinnet. Roles of Plant Small RNAs in Biotic Stress Responses. Annu. Rev. Plant Biol. 2009. 60:485–510. 10.1146/annurev.arplant.043008.092111

[4]

Feng Li and Shou-Wei Ding. Virus Counterdefense: Diverse Strategies for Evading the RNA- Silencing Immunity. Annu Rev Microbiol. 2006 ; 60: 503–531. doi:10.1146/annurev.micro.60.080805.142205.

[5]

Kriton Kalantidis, Heiko Tobias Schumacher, Tasos Alexiadis and Jutta Maria Helm RNA silencing movement in plants. Biol. Cell (2008) 100, 13–26 doi:10.1042/BC20070079

[6]

Juan A.D´ıaz-Pendo´n, andShou-WeiDing. Annu. Direct and Indirect Roles of Viral Suppressors of RNA Silencing in Pathogenesis.
Rev. Phytopathol. 2008. 46:303–26. 10.1146/annurev.phyto.46.081407.104746

[7]

Ahlquist, P., Janda, M. (1984). cDNA Cloning and In Vitro Transcription of the Complete Brome Mosaic Virus Genome. Molecular and Cellular Biology , 4 (12), 2876-2882.

[8]

Inmaculada González, Llucia Martínez, Daria V. Rakitina, Mathew G. Lewsey, Félix A. Atencio, César Llave, Natalia O. Kalinina, John P. Carr, Peter Palukaitis, and Tomás Canto. Cucumber Mosaic Virus 2b Protein Subcellular Targets and Interactions: Their Significance to RNA Silencing Suppressor Activity. MPMI Vol. 23, No. 3, 2010, pp. 294–303. doi:10.1094 / MPMI -23-3-0294.

[9]

Bujarski, J. J. (2008). Bromoviruses. In Mahy, B. W. J., Van Regenmortel, M. H. V. (Eds.), Encyclopedia of Virology (3rd ed.). (386-390). Elsevier Ltd.

[10]

Shou-Wei Ding', Wan-Xiang Li and Robert H.Symons.
A novel naturally occurring hybrid gene encoded by a plant RNA virus facilitates long distance virus movement. The EMBO Journal vol.14 no.23 pp.5762-5772, 1995

[11]

Douglas C. Prasher, Virginia K. Eckenrode, William W. Ward, Frank G. Prendergast, Milton J. Cormier. Primary structure of the Aequorea victoria green-fluorescent protein. Gene. Volume 111, Issue 2, 15 February 1992, Pages 229–233

[12]

K. Gopinath, B. Dragnea, and C. Kao. Interaction between Brome Mosaic Virus Proteins and RNAs: Effects on RNA Replication, Protein Expression, and RNA Stability. JOURNAL OF VIROLOGY, Nov. 2005, p. 14222–14234

[13]

Yuriy V. Sheludko. Agrobacterium-Mediated Transient Expression as an Approach to Production of Recombinant Proteins in Plants. Recent Patents on Biotechnology 2008, 2, 198-208

[14]

Zsuzsanna Mérai, Zoltán Kerényi, Attila Molnár, Endre Barta, Anna Válóczi, György Bisztray, Zoltán Havelda, József Burgyán and Dániel Silhavy. Aureusvirus P14 Is an Efficient RNA Silencing Suppressor That Binds Double-Stranded RNAs without Size Specificity. J. Virol. 2005, 79(11):7217. DOI: 10.1128/JVI.79.11.7217-7226.2005.

[15]

Moissiard, G. & Voinnet, O. (2004). Viral suppression of RNA silencing in plants. Mol Plant Pathol 5: 71-82

[16]

Dıaz-Pendon, J.A. & Ding, S-W.(2008). Direct and Indirect Roles of Viral Suppressors of RNA Silencing in Pathogenesis. Annual Review of Phytopathology, Vol.46, pp. 303– 26

[17]

Qu, Feng and Morris, Thomas Jack, "Suppressors of RNA Silencing Encoded by Plant Viruses and Their Role in Viral Infections" (2005). Virology Papers. Paper 125. 10-31-2005.

[18]

Charles W Melnyk, Attila Molnar and David C Baulcombe. Intercellular and systemic movement of RNA silencing signals. The EMBO Journal (2011) 30, 3553–3563.

[19]

Hamilton AJ, Voinnet O, Chappell L, Baulcombe DC. Two classes of short interfering RNA in RNA silencing. EMBO J 21: 4671–4679(2002).

[20]

Rui Lu, Alexey Folimonov, Michael Shintaku, Wan-Xiang Li, Bryce W. Falk, William O. Dawson, and Shou-Wei Ding. Three distinct suppressors of RNA silencing encoded by a 20-kb viral RNA genome. 15742–15747 PNAS November 2, 2004 vol. 101 no. 44.